Evaluation of genetic and strain specific factors on root colonization in endophytic insect pathogenic fungi (EIPF) *Metarhizium* and *Beauveria,* with special emphasis on hydrophobins

by

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Abstract

The Ascomycete genera, *Metarhizium* and *Beauveria*, are traditionally known as insect pathogenic fungi and are widely used as mycopesticides in agricultural settings. More recently, an additional role of these fungi in nature as root symbionts, with the ability to transfer nitrogen from dead insects to host plants, has been recognized. In this study, the genetic as well as strain-specific factors of *Metarhizium* and *Beauveria* were assessed during interaction with the plant and insect hosts. Hydrophobins are small proteins, unique to filamentous fungi, that provide hydrophobicity to aerial hyphae and conidia, and also supports fungal attachment to host surfaces. The role of two hydrophobins (hyd1 and hyd2*)* in insect pathogenicity were previously described in *Beauveria*, but little is known about their possible role in root colonization. Gene expression and plant root colonization assays revealed that the deletion of *hyd1* or *hyd2* subjected *Beauveria* to stress, which subsequently altered the expression of genes involved in signaling pathways, pigment production, specific adhesins, as well as fungal association with the root. The involvement of six *Metarhizium* genes on plant root colonization and insect pathogenesis were also investigated. Nitrogen transporter genes, *Mep2*, *MepC* and *Urease*, were selected due to sequence similarity with previously characterized plant-associating fungal ammonium transporters. Root colonization assays showed that the targeted deletion of *MepC* and *Mep2* genes in *M. robertsii* enhanced the rhizoplane colonization on barley roots and insect-derived nitrogen transfer to plant hosts. Three other genes were selected on the basis on RNA-Seq data that showed high expression levels on bean roots; these encoded a hydrophobin (Hyd3), a subtilisin-like serine protease (Pr1A) and a hypothetical protein. Root colonization assessment revealed that the loss of *Hyd3*, *Pr1A*, or the hypothetical protein gene from *M. robertsii* had no influence on establishing association with

barley roots. We also assessed ten *Metarhizium* generalist and specialist strains and a related endophytic fungus *Pochonia*, for insect pathogenicity and their ability colonize plants; however, regardless of whether the *Metarhizium* species was a generalist or specialist insect pathogen all strains tested showed some ability to associate with plants. Moreover, *Metarhizium* spp. were able to colonize monocots better than dicots. Our data indicates that even after divergence as generalist or specialist insect pathogens, *Metarhizium* spp. maintain their ancestral ability to colonize plants. Overall, this study provides useful insights into the genes involved in EIPF-root interactions and also highlights the impact of gene deletion in triggering compensatory pathways.

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List of Abbreviations

AM – arbuscular mycorrhizal fungi

AMT – ammonium transporters

CFU – colony forming unit

bad – *Beauveria* adhesin

BI – Bayesian interference

BRE – bean root exudate

BS – basal salt

cag8 – conidiation associated gene 8

CDEP1 – cuticle degrading protease

CTAB – cetyl trimethylammonium bromide

CTC – chloramphenicol-thiabendazole-cycloheximide

CZA- czapek-dox agar

EIPF – endophytic insect pathogenic fungi

GPCR – G-protein coupled receptor

IPF – insect pathogenic fungi

Hog1 – high-osmolarity glycerol

Hyd/hyd – hydrophobin

ITS – internal transcribed spacer

LT50 – median lethal time

Mad – *Metarhizium* adhesin

MAPK – mitogen-activated protein kinase

Mep – methyl ammonium permease

ML – maximum-likelihood

MMN - modified Melin-Norkrans

MrINV – *Metarhizium* invertase

Mrt – *Metarhizium* raffinose transporter

MYA – million years ago

NBT – nitro blue tetrazolium

NJ – neighbor joining

OA – orsellinic acid

PCR – polymerase chain reaction

PDA – potato dextrose agar

PHI – pathogen-host interaction

pks9 – polyketide synthase 9

pr1A – subtilisin like protaese1 A

ROS – reactive oxygen species

SDA – sabouraud dextrose agar

spp. – species

TF – transcription factor

WT – wild-type

YPD – yeast extract potato dextrose

Chapter 1 - Introduction

In most ecosystems, plants associate with endophytic fungi or mycorrhizal fungi to adapt to certain environmental conditions and to obtain otherwise inaccessible soil nutrients [1]. Endophytic fungi are classified by their ability to colonize internal tissues of plants without causing any disease symptoms. Association with certain endophytic fungi can be beneficial to plants including increased water-use efficiency, nutrient absorption, disease resistance and protection from biotic or abiotic stresses [2].

Metarhizium spp. (Clavicipitaceae) and *Beauveria* spp. (Cordycipitaceae) are facultative insect pathogenic fungi and are commercially used as mycopesticides in agricultural settings [3]. Recently, several members from these two genera were found to associate with plants either as endophytes or symbionts, and are termed endophytic insect pathogenic fungi (EIPF) [4]. These fungi have the ability to confer protection to plants against fungal pathogens [5] and also possess the ability to transfer nitrogen from infected insects to plants [6]. Because of these properties, EIPF have great promise in agriculture as biocontrol agents and as growth promoters. Presently, there is little information about the mechanisms involved in plant-EIPF communication during symbiosis and nutrient transfer. The overall focus of this thesis is to evaluate the role of different EIPF genes as well as how the evolutionary divergence of EIPF to insect pathogens affected the plant root colonization. In this study, we report the contribution of hydrophobins and nitrogen transporter genes during EIPF-root interaction. We also report that the variation in the insect pathogenicity among different *Metarhizium* spp. have no correlation with root colonization ability.

Hydrophobins are low molecular mass (7-9 kDa) secreted proteins unique to filamentous fungi. These proteins have an important role in fungal life cycles as a structural component during

growth and also to facilitate fungal interactions with the environment. These proteins help fungal aerial hyphae to grow into the air and also mediate the adhesion of fungal spores to hydrophobic surfaces [7, 8]. Furthermore, the importance of hydrophobins during fungal interaction with plant or animal hosts have been demonstrated in numerous fungal species [7]. Although the role of hydrophobins during insect infection was demonstrated, in both *Metarhizium* and *Beauveria* [9, 10], little is known about the possible role of hydrophobins during their interaction with plants. One of the main objectives of this study was to investigate the role of EIPF hydrophobins on the initial interaction with plant roots.

Recent studies demonstrated the ability of *Metarhizium* to transfer insect derived nitrogen to plant hosts [6], however, the specific transporters involved in this transfer are still unknown. Another objective of this study was to understand the role of nitrogen transporters present in *Metarhizium* during symbiotic association with plants. We also examined the transcriptome of *Metarhizium*-colonized bean roots to understand the expression pattern of fungal genes during plant-fungal symbiosis.

Metarhizium diverged from other Clavicipitacean endophytes or plant symbionts (*Epichloe* and *Claviceps*) approximately 100 MYA and subsequently acquired the ability to infect insects. [11]. *Metarhizium* spp. vary greatly with respect to insect host range. Some *Metarhizium* species have a wide host range, infecting and killing over 200 insect species, while others possess a narrow host range, infecting only a few insect species. *Metarhizium* spp. with broad insect host range are known as generalist and those species with narrow host range are known as specialist insect pathogens [12]. The divergence between generalist and specialist strains occurred more recently approximately 35 MYA [11]. However, there are few studies done to identify whether different *Metarhizium* strains exhibit a specialist or generalist host range during plant colonization. The

third objective of this thesis is to understand the range of root colonization by specialist and generalist insect pathogen species or the colonization preferences toward any particular plant species.

1.1 Outline

Chapter 2 provides a literature review describing the current knowledge on evolutionary, ecological, molecular genetics, and applied aspects of the EIPF, *Metarhizium* and *Beauveria*.

Chapter 3 is a manuscript published in *Microbiology* in 2018. This chapter discusses the involvement of hydrophobins in *Beauveria bassiana* on association with plant hosts. In addition, the pleiotropic consequences of hydrophobin gene deletion on fungal phenotype, signaling and stress pathways, as well as the ability of *B. bassiana* to associate with plant roots, were also investigated.

Chapter 4 demonstrates the genetic aspects of *Metarhizium*-plant interactions using targeted gene deletion fungal strains. This chapter investigates the role of three *M. robertsii* nitrogen transporter genes (*MepC*, *Mep2*, and *Urease*), during association with barley roots. These three genes were selected since homologous genes in arbuscular mycorrhizal fungi were reported to play a pivotal role in nitrogen mobilization during plant root colonization. In addition, the role of three other genes, *hydrophobin 3 (Hyd3)*, *subtilisin-like protease* (*Pr1A*) and a *hypothetical protein (Hypo. Protein)* were also evaluated. These genes were included on the basis of RNA-Seq data as these genes showed high expression levels on colonized bean roots. The root colonization assay showed that the deletion of two ammonium transporters in *M. robertsii*, *MepC* and *Mep2* had an influence on barley root rhizoplane association and the transfer of insect derived nitrogen to plant hosts. Remarkably, the fungal genes (*Hyd3*, *Pr1A* and *Hypo. protein*) that were upregulated on symbiotic association with plant roots, had no direct correlation with plant root

association. In regard to the insect pathogenicity of these mutants, *Hyd3* and *Pr1A* mutants showed delayed mortality against one of two insect species tested. However, the targeted deletion of other genes (*MepC, Mep2, Urease* and *Hypo. protein*) did not reduce the fungal pathogenicity against insect hosts.

Chapter 5 presents the strain-specific aspects of EIPF concerning root colonization and insect pathogenesis. In this chapter, the ability to form endophytic, rhizoplane, and rhizospheric associations with monocot and dicot roots was assessed for specialist and generalist *Metarhizium* spp., as well as a related fungus, *Pochonia chlamydosporia*. In addition, insect pathogenicity, growth rate, and conidial hydrophobicity of *Metarhizium* spp. and *P. chlamydosporia* were also evaluated. Here we report that regardless of differences in insect host range, *Metarhizium* species and *Pochonia* can form associations with plant roots. Remarkably, *Metarhizium* spp. and *Pochonia* extensively colonized monocot roots endophytically compared to dicot roots. All strains exhibited similar conidial hydrophobicity indices. *M. frigidum* had the slowest growth rate on PDA, however there were no differences in colonizing roots relative to faster growing *Metarhizium* strains. The data from this study indicates that different *Metarhizium* spp. can form associations with plant species and the divergence with insect host specificity is not correlated with root colonization ability. This suggests that even after evolutionary divergence as specialist or generalist insect pathogens, different *Metarhizium* spp. still retained an ecological niche as plant symbionts.

Chapter 6 presents the general discussion of the research as well as the main conclusions of this study and future directives.

1.2 Co-Authorship: Described below are the contributions from each author to multi-authored chapters.

Chapter 2: Insect pathogenic fungi as endophytes is a review paper published in *Advances in Genetics* (Vol. 94, pp. 107-135), Academic Press (2016). The manuscript was written by Moonjely S, Barelli L and Bidochka MJ.

Chapter 3: Hydrophobins contribute to root colonization and stress responses in the endophytic insect pathogenic fungus *Beauveria bassiana.* The work from this chapter has been published in *Microbiology*, 2018 (doi: 10.1099/mic.0.000644). The manuscript was written by Moonjely S, Keyhani NO and Bidochka MJ. Bidochka MJ and Moonjely S were responsible for experimental design. Moonjely S performed the experiments.

Chapter 4: *Metarhizium robertsii* ammonium permeases (MepC and Mep2) contribute to rhizospheric colonization and modulates the transfer of insect derived nitrogen to plants. The work from this chapter has been submitted to *Plos One* (March 2019). The manuscript was written by Moonjely S, Zhang X, Fang W and Bidochka MJ. Bidochka MJ and Moonjely S were responsible for experimental design. Zhang X created the targeted gene deletion fungal strains and performed the phytogenetic analysis of MepC and Mep2. Moonjely S performed phenotypic analysis, root colonization assay, insect pathogenesis assay, ^{15}N -transfer experiment, nitrogen source assay.

Chapter 4: Generalist and specialist *Metarhizium* insect pathogens retain ancestral ability to colonize plant root. The work from this chapter has been submitted to *Fungal Ecology* (January 2019). The manuscript was written by Moonjely S and Bidochka MJ. Moonjely S performed the experiments.

Chapter 2- Literature review
Insect pathogenic fungi as endophytes

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2.1 Abstract

In this chapter, we explore some of the evolutionary, ecological, molecular genetics, and applied aspects of a subset of insect pathogenic fungi that also have a lifestyle as endophytes and we term these endophytic, insect pathogenic fungi (EIPF). We focus particularly on *Metarhizium* spp. and *Beauveria bassiana* as EIPF. The discussion of the evolution of EIPF challenges a view that these fungi were first and foremost insect pathogens that eventually evolved to colonize plants. Phylogenetic evidence shows that the lineages of EIPF are most closely related to grass endophytes that diverged ≈ 100 MYA. We discuss the relationship between genes involved in "insect pathogenesis" and those involved in "endophytism" and provide examples of genes with potential importance in lifestyle transitions toward insect pathogenicity. That is, some genes for insect pathogenesis may have been co-opted from genes involved in endophytic colonization. Other genes may be multifunctional and serve in both lifestyle capacities. The interactions of EIPF with their host plants are discussed in some detail. The genetic basis for rhizospheric competence, plant communication, and nutrient exchange is examined, and we highlight, with examples, the benefits of EIPF to plants, and the potential reservoir of secondary metabolites hidden within these beneficial symbioses.

2.2 Introduction

Insect pathogenic fungi (IPF) encompass over 1000 fungal species found in most major fungal taxonomic groups from Chytridiomycetes to Basidiomycetes. However, phylogenetic data provide little evidence for a single common origin for insect pathogenesis even within a major taxonomic group [13, 14]. There is also a wide range of host specificity in IPF. For example, some fungal species within the Entomophthoromycota (e.g., *Entomophthora*, *Strongwellsea*, and *Entomophaga*) are obligate pathogens of a single or very few taxonomically related insect species. Even within a single genus there is a wide range of host specificity. For example, within the hypocrealean fungal genus, *Metarhizium*, there are facultative pathogens with a wide range of host insects (e.g., *Metarhizium robertsii* and *M. brunneum*), as well as species with a narrow host range (e.g., *M. acridum* and *M. flavoviride*) [15]. Besides the host range, generalist and specialist species follow two different virulence strategies against insect hosts; generalists species kill insects by producing toxins, whereas specialist species causes systematic infections in hosts before death [11, 16].

IPF generally infect their host through transcuticular penetration and need not be ingested. Once they enter the insect hemocoel, they ramify within the insect. After that, fungal hyphae emerge from the insect. The outgrowth of fungal structures from infected, mummified insects is the stuff of science fiction. These fungi serve as models for the mechanics, molecular biology, and evolution of pathogenesis [14, 17]. Furthermore, the potential of agricultural applications of *Metarhizium* and *Beauveria* for insect biocontrol has been known since the early 1900s [18, 19], and there are many commercially available formulations [15, 20]. Several IPF are also being developed as pathogens of human disease vectors. For example, natural and transgenic strains of

Metarhizium are being explored as pathogens of mosquitoes in order to combat malaria and other diseases [21–24].

Adding further ecological and evolutionary complexity and intrigue to a subset of hypocrealean IPF is a multifunctional lifestyle that includes their role as endophytic symbionts of plants. Two model IPF that have also demonstrated endophytic capability are *Metarhizium* and *Beauveria* [17, 25, 26]. The genus *Metarhizium* is particularly interesting as an evolutionary model since, as previously mentioned, not only does this genus represent species with narrow and broad insect host ranges, but they also vary in their endophytic capabilities.

2.3 Evolution of endophytic insect pathogenic fungi

Phylogenetic studies have shown that species of IPF, such as *Metarhizium*, *Beauveria*, *Lecanicillium*, and *Paecilomyces*, are most closely related to the fungal grass endosymbionts *Claviceps* and *Epichloë* [27, 28]. In addition, comparative genomic analyses proposed that the *Metarhizium* lineage diverged from the lineage of the plant endophyte *Epichloë festucae* approximately 88-114 MYA. *Metarhizium* and *Beauveria* are more closely related to endophytes and plant pathogens than to animal pathogens. The genome of *Metarhizium* shows a large number of genes for plant-degrading enzymes [11]. This evidence supports the idea that the *Metarhizium* lineage evolved from plant-associated fungi, and insect pathogenicity is a more recently acquired adaptation. Hypothetical mechanisms by which genes involved in insect pathogenesis may have arisen have been theorized as being coopted, evolved, or acquired by horizontal gene transfer from other fungi or host insects [29].

The evolution of IPF must have involved adaptations that allowed degradation of insect cuticle and host body components, as indicated by the large number of proteases, lipases, and

chitinases present within genomes of endophytic IPF (EIPF). The mechanisms of insect pathogenicity, and how genes involved in insect pathogenicity are related to those involved in endophytism. Furthermore, refinement of mutualistic plant colonization and the loss of genes involved in plant pathogenesis would have also been driving factors in the evolution of *Metarhizium* as an endophyte [30].

Interkingdom host jumping by EIPF from plants back to arthropods and then back to plants has been proposed for the evolution of insect pathogenicity [13]. In contrast, we suggest that many of these fungi evolved to infect insects while maintaining their mutualistic endosymbiosis with plants. We cannot reenact the ecological conditions that allowed for niche diversification by the ancestors of these endophytic fungi, as they evolved insect pathogenic capabilities c. 100 MYA. However, we hypothesize that the driving force behind this evolution was the host plant demanding reciprocal nutrient exchange from the fungus in exchange for access to plant carbohydrates in the rhizosphere. IPF would be able to provide the plant with a source of nitrogen, or other growth-limiting nutrients, derived from insect parasitism.

2.4 Multifunctional lifestyles

2.4.1 Insect Pathogenicity

IPF are ubiquitous in nature, and they range from narrow host range to broad host range. The underlying molecular mechanisms of the infection process are well studied and documented particularly for *Metarhizium* spp. and *Beauveria bassiana* [3, 31, 32]. These fungi have been established as model organisms for general infection processes. In the following, we relate mechanisms of insect pathogenesis in light of the evolutionary history of these fungi as plant

associates. That is, are there similarities in genes involved in insect pathogenesis and those involved in plant associations?

2.4.1.1 Adhesion

The conidia of *Metarhizium* and *Beauveria* are generally hydrophobic, and adhesion of these fungi to the host insect cuticle is mediated by means of specific adhesion genes or nonspecific (principally hydrophobic) interactions. The surface structure and composition of the insect exoskeleton influence the adherence of fungal conidia to the cuticle. The outermost layer of the insect integument is a lipid layer, which is hydrophobic in nature, facilitating attachment of fungal propagules [33, 34]. In *Metarhizium* spp., conidial adherence to the insect cuticle is facilitated by both specific and nonspecific interactions.

The key genes involved are the adhesin-like protein, *Mad1*, and *ssgA/HYD1*, a hydrophobin [35–37]. Two other hydrophobin genes, *HYD3* and *HYD2*, which code for class I and class II hydrophobins respectively, have been reported in *M. brunneum* and aid in conidial adhesion to insect epicuticle and affect virulence [9]. In *B. bassiana*, the initial adhesion to insect cuticle is mediated via nonspecific hydrophobic interactions. Studies have shown that in *Beauveria* two hydrophobin genes, *hyd1* and *hyd2* encode for class I hydrophobins and play a significant role in adhesion and virulence [10].

It is interesting, from an endophytic perspective, that the mechanisms of insect infection and plant infection show remarkable similarities. Comparative analysis of the *Metarhizium* hydrophobin genes, using the pathogen host interaction (PHI) gene database, has shown marked similarity with sequences from plant pathogenic fungi [11]. For example, hydrophobins have also been implicated in plant infection processes in the rice blast fungus, *Magnaporthe grisea* [8]. In particular, the *M. grisea* class I hydrophobin gene, *Mpg1* acts as an adhesion factor initiating the

infection process in plants [8], similar to the role of *ssgA* (hydrophobin; *Metarhizium*) enabling adherence to insect cuticle [37].

2.4.1.2 Penetration

The insect integument is formed of several layered epicuticle, procuticle, and epidermis, which are composed mainly of lipids, chitins, and proteins. The conidial surface proteins act synergistically to aid in germination through recognition of insect-specific components and subsequent cuticle degradation [3, 34, 38]. The major carbon sources utilized for conidial germination are endogenous or cuticular lipids [3, 34, 39, 40].

Once the fungal conidia successfully adhere to the insect cuticle, they germinate to form hyphae on the insect cuticle and express hydrolytic enzymes, such as proteases, esterases, N-acetylglucosaminidases, chitinases, and lipases [41–43]. In addition to enzymatic degradation, mechanical pressure through formation of specialized hyphal structures (appressoria) has also been implicated for successful cuticular penetration. For example, the expression of Mpl1 (perilipin) plays a significant role in the transport and breakdown of endogenous lipids that increases turgor pressure and thereby aids the formation of appressoria [44].

The sequential expression of different degradative enzymes in combination with mechanical pressure accelerates cuticular penetration by fungal hyphae toward the insect hemocoel [3, 31, 34, 39, 40, 45]. Transcriptomic analysis revealed that *Metarhizium* spp. express a diverse array of secreted proteases, including subtilisin-like proteases, trypsins, carboxypeptidase, aspartic protease, threonine protease, cysteine protease, and metalloproteases as penetration commences [11]. However, generalist and specialist strains of *Metarhizium* (*M. robertsii* and *M. acridum*,

respectively) express different proteases on the cuticle of their hosts, implying a role for proteases in virulence, host recognition, and in the different stages of pathogenesis.

The expression of high amounts of proteases presumably enables *Metarhizium* to adapt to different habitats [11]. Studies have shown that different proteases expressed by *Metarhizium* are important pathogenicity determinants [45]. Gao et al. (2011) also reported that *M. robertsii* expresses many more trypsins than pathogens of plants, animals, or fungi. Screening of trypsins from *Metarhizium* using the PHI database of phytopathogen virulence determinants revealed a similarity with glucanase-inhibitor proteins (GIP), a trypsin from the soybean pathogen *Phytophthora sojae* [11]. *Phytophthora sojae* secretes GIP when infecting plants and thereby reduces the induction of plant-derived defense responses [46]. These studies provide important insights into mechanisms of insect pathogenesis, but genetic similarities to plant pathogens and endophytes tempt further investigation into the multifunctional roles of these genes, particularly in an endophytic capacity.

2.4.1.3 Proliferation, immune avoidance, and insect death

Through the combination of mechanical pressure and enzymatic processes, fungal hyphae penetrate the insect cuticle and eventually reach the insect hemocoel, where they differentiate to form yeast-like bodies called blastospores. Insect hemolymph is rich in nutrients with the most abundant carbohydrate in hemolymph being trehalose [31].

Upon reaching the insect hemolymph, the fungal hyphae switch phenotypes to blastospores and short hyphal lengths called hyphal bodies [47, 48]. *Metarhizium* expresses a collagen-like protein (mcl1) which functions as a defensive coat that prevents hyphal bodies from being

phagocytosed or encapsulated by host immune cells [49], however, gene-sequencing studies have shown that a homolog of the collagen-like protein is absent in *Beauveria* [50].

Metarhizium and *Beauveria* can also survive phagocytosis by amoeboid predators in the soil [51]; a survival strategy also exhibited by several mammalian fungal and bacterial pathogens. This suggests that the ability of fungal insect and mammalian pathogens to survive host phagocytic cells may be a consequence of adaptations that originally evolved in order to avoid predation by soil amoeba. This potentially speaks to strategies of survival of these EIPF fungi as they persist in the rhizosphere as endophytes [52].

Beauveria and *Metarhizium* also produce insecticidal metabolites, including beauvericin and destruxins, respectively, that allow blastospores to proliferate inside the hemolymph [31]. Besides cuticle degradation, proteases are involved in nutrient acquisition, degradation of antifungal proteins to bypass the host immune response, and in the regulation of microenvironmental pH [53].

2.4.1.4 Conidiation on the surface of the insect cadaver

Blastospores proliferating within the hemolymph kill the insect host within 3-7 days by absorbing hemocoelic nutrients and through toxic metabolite production [41, 48]. After fungal hyphae ramify throughout the dead infected host, they reemerge from the insect and conidiate on the insect cadaver. *Metarhizium* produces green conidia on the surface of the insect cadaver, from which the designation of "green muscardine disease" arose; *Beauveria* infections result in white conidia. Suppression subtractive hybridization has shown significant upregulation of the gene, *cag7* (*Pr1 protease*) in *Metarhizium* at the onset of conidiation [48].

The complex process of insect pathogenesis requires these fungi to undergo morphological switching from conidia to hyphae, to appressoria, to single-celled blastospores, and finally conidiogenous cells, all of which utilize overlapping subsets of genes. Furthermore, these fungi are constantly sampling the environment and relaying those messages through signaling mechanisms for gene transcription. Microarray analysis has shown that in *Metarhizium* and *Beauveria*, overlapping subsets of genes are differentially expressed when they are grown in media containing insect cuticle, insect hemolymph, or plant root exudate. The abundance of proteases, and the differential expression of genes by *Metarhizium* in particular reveals the phenotypic plasticity that enables this fungus to survive both as an insect pathogen and as a plant endophyte [11, 54].

2.4.1.5 Proteins and signaling mechanisms involved in insect pathogenesis

A number of membrane proteins, transcription factors (TFs), and biochemical pathways implicated in insect pathogenesis have been characterized in the model IPF *Metarhizium* and *Beauveria* [14, 47]. Tetraspanin, a membrane protein involved in membrane signaling, is required to breach the insect cuticle, as *M. acridum* mutants impaired for tetraspanin (*pls1*) showed reduced virulence [55]. Tetraspanin appeared to be involved in the initial infection stages including conidial germination, formation of appressoria, and production of cuticle-degrading enzymes [55].

Msn2, a well-characterized stress response TF in yeast, has also been identified in *Beauveria* and *Metarhizium* and is crucial for virulence, conidiation, and the stress response; a deletion mutant showed a marked repression of diverse virulence-associated genes [56]. Functional characterization found that a basic leucine-zipper domain of the *M. robertsii* TF MBZ1 is essential for proper functionality, as gene deletion resulted in decreased virulence toward wax

moth larvae [56]. Additionally, MBZ1 positively regulates the adhesin gene *Mad1*, as the expression of *Mad1* was found to be downregulated fourfold in *Metarhizium MBZ1* deletion mutants [57]. Characterization of a homolog of the PacC TF (pH-responsive TF) in *Metarhizium* showed that PacC contributes to fungal virulence by impacting cuticle penetration, evasion of the host immune response, and mycosis [58]. Mutant strains lacking a functional PacC gene were found to be unable to penetrate the insect cuticle and proliferate in the hemocoel [58]. In terms of virulence, two other TFs characterized in *B. bassiana* are CreA [59] and a multiprotein binding factor (MBF1) [60].

Several of the signaling pathways that contribute to the virulence of IPF have been characterized in both *Metarhizium* and *Beauveria*. These include G-protein-coupled receptor (GPCR) signaling, mitogen-activated protein kinase (MAP kinase), and cAMP-PKA pathways. Fungal GPCRs are involved in niche recognition and nutrient sensing. Insect bioassays using *Beauveria* mutants defective in the GPCR3 gene revealed reduced virulence on topical application, indicating the role of GPCRs in initial infection stages [61]. Targeted gene disruption of the *Metarhizium anisopliae* regulators of G-protein signaling (RGS) gene, *cag8* (conidiation associated gene) showed that *cag8* plays a significant role in virulence and hydrophobin synthesis [62]. The role MAP kinase pathways play in insect virulence has been studied in *M. robertsii* and *B. bassiana* through genetic analysis. Targeted deletion of the *MAPK1* gene in *B. bassiana* demonstrated that MAPK1 protein is involved in fungal adhesion and penetration of insect cuticle [63]. Characterization of another MAPK in *B. bassiana* encoding Bb *hog1* showed the key role of functional HOG1 MAPK in pathogenicity toward insects [64]. Similarly, *M. acridum* mutants defective for hog1 MAPK signaling showed greatly reduced virulence [65]. *Beauveria bassiana slt2* encodes a *slt2* family MAPK and is likewise involved in insect virulence. Mutant strains that

lack *slt2* show a marked decrease in virulence in insect bioassays using either topical application or hemocoel injection of spores [66]. Targeted gene expression studies with *Beauveria* defective for adenylate cyclase, the key enzyme that catalyzes the conversion of adenosine triphosphate (ATP) to 3',5'-cyclic adenosine monophosphate (cAMP), showed that cAMP-dependent processes were crucial for virulence [67]. Fang *et al* (2009) investigated the role of the cAMP-dependent protein kinase A (PKA) subunit in insect pathogenesis and reported that *M. robertsii* deficient in the pka1 subunit were greatly reduced in virulence (>90%) against wax moth larvae. Moreover, microarray analysis on mutant strains that lack the pka1 subunit showed downregulation of 244 genes involved in cuticular infection process [68].

Despite infection processes being similar overall for *Metarhizium* and *Beauveria*, there are differences in key molecular aspects that arbitrate virulence toward insect hosts [47]. MAP kinases and other signaling mechanisms, homologous to those in IPF, are well known in plant pathogenic fungi [69], and our knowledge of the molecular interplay between plant and microbial symbionts has developed over the last decade [70]. The signaling mechanisms that have been studied for insect pathogenesis in IPF could have the same or parallel pathways in their endophytic capacities.

2.5 Relationship between insect pathogen genes and endophytism

The endophytic abilities of these fungi add a level of complexity to their role as insect pathogens. In light of this, the genomes of these fungi are extremely interesting [14]. There needs to be a change in the approach to the analysis of these organisms, their genetic makeup, and their ecological interactions as insect pathogens and plant associates. For example, the mutants in signaling genes discussed in the previous chapter should also be analyzed for their endophytic capacities.

The multifunctional lifestyles of these fungi require genotypic plasticity when exposed to diverse environments for survival. For example, to establish adherence to insect cuticle or plant roots, *Metarhizium* differentially expresses the adhesin proteins MAD1 and MAD2, respectively [37]. Tracing genes pertinent to all lifestyles, those that overlap in functionality, would help to elucidate the evolutionary history of these fungi. Another example is the subtilisin-like protease, Pr1A, from *Metarhizium* that is highly expressed in media of both insect and plant origin [71]. It has been suggested that adaptation to various hosts is the result of gene duplication events or horizontal gene transfer [29, 50, 72]. Further investigations of the molecular mechanisms for the symbiosis between plants and EIPF will help to uncover the genetic events that lead to insect pathogenicity. For example, the involvement of the subtilisin-like family of *Metarhizium* proteases in endophytism may have preadapted the ancestor of *Metarhizium* to insect pathogenesis. If we accept the hypothesis that some IPF evolved from plant symbionts, from where did the genes involved in insect pathogenicity evolve? As an example, the protease, Atl1, from the grass endophyte *Acremonium typhinum*, facilitates fungal colonization by assisting in the degradation of the plant cell wall [73]. Homologous proteases have been identified in species of the EIPF fungi *Metarhizium* and *Beauveria*, the mycoparasite *Trichoderma harzianum*, and the nematode-trapping fungus *Arthrobotrys oligospora*, all of which are also endophytes [73–75]. It is possible that the IPF proteases had initially been utilized for endophytic colonization and were subsequently coopted for their utility in insect pathogenesis. This may be particularly true for the subtilisin-like proteases found in *Metarhizium* that may have arisen by gene duplication events [76, 77] from an ancestral "endophytic protease." Since some of these IPF are also endophytes, delving into the evolutionary relationships between "insect pathogenic" genes and "endophytic" genes in a single organism could be extremely informative. Examples previously mentioned are the subtilisin-like

protease genes and the adhesin genes, but that is probably the tip of the iceberg and many more genes could be identified. What, if any, are the relationships between these genes? Did they arise by duplication events and subsequent specialization? Or were they gained through horizontal gene transfer? If so, what was the potential source?

2.5.1 Plant root colonization by insect pathogenic fungi

Traditionally IPF were considered solely as insect pathogens, but are now also being investigated as endophytes [2, 25, 78] and rhizosphere colonizers [79–81]. This dual lifestyle provides promising opportunities for EIPF, not only as biocontrol agents, but also as biofertilizers and for general plant protection. Why adopt a dual role as insect pathogens and endophytes? What drove evolution from a plant colonizer to an insect pathogen? Previously, we showed that plants were able to reacquire nitrogen from insects through a partnership with the endophytic, insect pathogenic fungus *M. robertsii* [6, 28]. That is, the endophytic capability and insect pathogenicity of *M. robertsii* and *B. bassiana* are coupled so that these fungi act as conduits to provide insect-derived nitrogen to plant hosts. We suggest that EIPF initially evolved as plant colonizers and that this relationship was encouraged by the ability of these fungi to provide nitrogen, or other nutrients, first as nutrient scavengers, then through adaptations as insect pathogens. The consequence of this is that plants forming relationships with IPF would have had an advantage, particularly in nutrient poor soils.

The role of *Metarhizium* and *Beauveria* as insect pathogens and endophytes results in healthier plants [25, 82–84]. The discovery of this lifestyle as plant colonizers has led to a reevaluation of the ecology of other IPF. For example, *Ophiocordyceps sinensis* is a pathogen specific to larvae of the ghost moth *Thitarodes* [85]. It is believed that without specific nutrients

from the larval tissue, *O. sinensis* cannot complete the teleomorph stage of its life cycle [85], and the genome of *O. sinensis* has lost many of the genes that allow *Metarhizium* spp. to interact with plants [86, 87]. However, Zhong et al. [88] detected *O. sinensis* on the roots of as many as 23 different plants. The finding that such a host-specific IP fungus can also colonize plants suggests that numerous IPF play larger roles in their ecosystems than previously thought, and that plant host range may influence evolution of these IPF [28]. We have previously suggested that host specific IPF may not be capable of forming associations with plants since there would be selective pressure toward host insect specificity [28]. However, the occurrence of *O. sinensis* on the roots of numerous plants suggests that even pathogenic fungi with a very narrow host range to insects can form plant associations. The type of association with the host plant(s) of *O. sinensis* is currently unknown (e.g., asymptomatic, beneficial, pathogenic, or, quite possibly, coincidental) but may be potentially beneficial as in several other plant-associating IPF (e.g., *Metarhizium*, *Beauveria*) [6, 89].

Although the complex interactions and ecology of the soil is still largely a "black box," isolation of fungal propagules from rhizospheric soil is potentially a strong indication of rhizospheric competence [32, 52]. The rhizosphere (the region of soil influenced by root chemistry) is a competitive habitat for nutrients as many nutrients are limited by being bound in unusable forms, requiring enzymatic digestion for absorption [90]. Survivability within the rhizosphere is also dictated by the ability of an organism to overcome abiotic stressors, such as temperature, pH, osmotic stress, and salinity [91]. Identifying and modifying genes responsible for tolerance to such stresses could allow increased survivability of IPF within soil. Conidia of a genetically engineered strain of *M. robertsii* overexpressing heat shock protein 25 (HSP25) had increased tolerance to extreme temperature and osmotic stress [83]. However, the survivability of

this engineered strain of *Metarhizium* was dependent on the presence of plant roots as the survivability within bulk soil was unaffected, but was increased in rhizospheric soils [83]. This highlights the fact that natural environmental conditions must be taken into account when determining the effectiveness of genetically engineered strains.

The ability of a fungus to persist in the rhizosphere is directly influenced by the plant root exudate and microbial diversity within the rhizosphere [92]. The types of compounds found in root exudate, although categorically similar (i.e., they all contain carbohydrates, amino acids, vitamins, etc.), differ in their proportions and specific makeup, and will alter the pH, organic matter composition, and thus the microbial community [93]. Several IPF have been shown to endophytically associate with plant roots and/or aboveground tissues (e.g., *Metarhizium*, *Beauveria*, *Lecanicillium*) [2, 6] and benefit the plant in many ways. Soil sampling in a region of Ontario, Canada, revealed three species of *Metarhizium*, *M. robertsii*, *M. brunneum*, and *Metarhizium guizhouense*, that associate with grasses, shrubs, and trees, respectively [94], and five species of *Metarhizium* were able to transfer insect nitrogen to monocots as well as dicots [6]. The ability of an EIPF to colonize a plant host would be dictated by its compatibility with the rhizospheric conditions created by root exudate and its ability to compete within this niche. Thus, any conclusions made that an IP fungus is unable to persist as a plant endophyte must come from exhaustive examination of a range of potential plant hosts, as some IP fungal strains may be more restricted than others in plant host range. For instance, *T. harzianum*, a notable mycoparasite that is also able to infect insects, was deemed rhizosphere-incompetent when evaluated for colonization of cucumber and radish roots [95]. However, *T. harzianum* is fully capable of colonizing and promoting the health of maize plants and is routinely used as a biopesticide against plant pathogenic fungi [96, 97]. Rhizospheric competence is a desirable attribute for a biopesticide, as

the ability of it to persist in the environment long after application would help to maintain crop protection throughout the year and minimize application costs. In order to manipulate naturally occurring endophytes, IPF genomic analyses are being conducted to elucidate genes specific to initiation and maintenance of plant-fungal symbioses [81], and certain fungal genes have already been identified as being essential for plant root symbiosis.

The *Mad2* gene of *M. robertsii* encodes a plant adhesin that is essential for attachment to plant material and is upregulated in bean root exudate [37]. The gene *Mrt*, encoding an oligosaccharide (raffinose) transporter, was found to be required for rhizospheric competence of *M. robertsii*, as deletion-mutant germlings failed to develop branching hyphae, and deletion mutants produced 11-fold fewer colony-forming units in rhizospheric soil than the wild type [98]. The role of this sugar transporter is clearly significant to the ability of *Metarhizium* to maintain rhizospheric competence. Gene knockout strains of *M. robertsii* for invertase (MrINV) resulted in severe reduction of growth in root exudates, but improved colonization on roots. The increased colonization could be due to lower availability of sugar in invertase mutants reducing carbon catabolite repression of enzymes that allow the fungus to colonize roots [99]. Carbohydrates are the most abundant component of root exudates [92], and could therefore have the biggest impact on the metabolic requirements and limitations of an EIPF.

Although *Metarhizium* appears to be restricted to colonization of plant roots, other EIPF are able to colonize aboveground tissues of plants as well. In an experiment with haricot bean, *M. robertsii* was found to localize to the root system below the hypocotyl in comparison to *B. bassiana* that was isolated from the roots, hypocotyl, stem, and leaves, after 60 days of growth [79]. The finding that *Beauveria* can colonize all tissues of a plant has been seen with tomato, cotton, corn,

and snap bean as well [26, 80], and the mechanism of entry is similar to that utilized for insect infection [26, 100].

The mechanism of plant colonization for many EIPF species is unknown, as the discovery of this ability is still relatively recent. How is a plant able to distinguish between a potentially beneficial partner and a pathogenic one? Early recognition events must take place as timing of the initiating defense mechanism is believed to be the difference between infection and resistance to a potential pathogen [101], and the initial phases of infection and colonization of pathogens, mutualists, and commensals are identical for many fungi [102]. A recent discovery with the arbuscular mycorrhizal fungus, *Glomus intraradices*, revealed a diffusible factor that stimulated root hair development in the legume, *Medicago truncatula* [103]. The structure of this communication molecule was determined to be the same as Nod factors released from rhizobia, a lipochitooligosaccharide (LCO). Molecules released from mycorrhizal fungi that prime the plant for colonization, termed myc factors, often induce activation of the SYM (symbiotic) signaling pathway to cause morphological changes (e.g., root hair growth) that increase contact between roots and fungus [104, 105]. In cultures of switchgrass inoculated with conidia of *Metarhizium*, there was extensive root hair development that was not observed in fungal-free cultures, indicating that *Metarhizium* is able to communicate to the plant prior to colonization, possibly with a myc-like factor [25]. As these LCOs appear to be utilized by both rhizobia and mycorrhizal fungi, it is a fair assumption that EIPF may utilize a similar molecule for communication to their host plants to distinguish themselves as beneficial symbionts.

2.5.2 Tripartite interactions of endophytic insect pathogenic fungi

EIPF potentially colonize plants in order to exploit a carbon source. In mycorrhizal symbioses, plants exchange photosynthetically derived carbohydrates for nutrients that would otherwise be unavailable for uptake (i.e., nitrogen and phosphorus) [106–108]. The EIPF *M. robertsii* was recently shown to provide its plant hosts with insect-derived nitrogen [6], and in return *Metarhizium* receives plant-derived carbohydrates [98]. This represents a previously unknown method of nitrogen acquisition for plants, as well as defining the association between the EIPF *M. robertsii* and its plant host as mutualistic. A question that remains is whether or not a continued symbiotic relationship, between plant and EIPF, is specifically reliant on nutrient exchange. That is, if the fungus were to suddenly stop transferring nitrogen, would the plant perceive the fungus as pathogenic and eject the fungal partner? In mycorrhizal symbioses between *Medicago* and various species of *Glomus*, the exchange of phosphorus and carbon is bidirectionally controlled [109]. Kiers et al. [109] tested the cooperation of both the plant and fungal partner in maintaining a successful symbiosis when each was supplied differing concentrations of nutrients for exchange. The results showed that both *Medicago* and *Glomus* were able to detect the nutrients provided by the partner and allocate the reciprocal resource based on the most rewarding root/hyphae. To this end, the ability of a plant to shift resource allocation to reciprocating hyphae and thus away from less-cooperative hyphae would limit the growth of the fungal symbiont and its survival in the absence of providing nutrients. This same mechanism was seen with regard to both nitrogen and carbon exchange [110]. The dynamics that sustain the association between facultative EIPF and plants, as compared to obligate mycorrhizal symbionts, may reflect similar control mechanisms, but this remains to be determined. If nutrient transfer were as tightly controlled in a bidirectional fashion with EIPF, it would lend support to the hypothesis

that fungal evolution toward insect parasitism occurred to increase the competitive advantage by permitting access to a larger, insect derived supply of nitrogen available in exchange for plant carbohydrates.

2.6 Application of endophytic insect pathogenic fungi

2.6.1 Insect pathogenic endophytes as biocontrol agents

Insect pest control in an agricultural setting has historically relied heavily on chemical pesticides; however, pest control practices have been shifting from chemical insecticides to the use of biological control agents [111]. Among these, IPF of genera including *Metarhizium*, *Beauveria*, *Lecanicillium*, *Isaria*, *Sporothrix*, *Hirsutella*, *Aschersonia*, *Paecilomyces*, *Tolypocladium*, and *Nomuraea* have been traditionally known and extensively studied as insect pest control agents in agriculture [15, 111]. Of these *Metarhizium*, *Beauveria*, *Lecanicillium*, and *Isaria* are among the most commercially available insect pathogens [111].

Recent work has specifically focused on genetic engineering to increase overall fungal virulence. For example, Wang and St. Leger (2007) genetically modified *M. robertsii* to express an insect-specific neurotoxin, a gene harvested from the scorpion, *Androctonus australis* [112]. Compared to the wild-type fungus, the neurotoxin-expressing strain reduced the survival time of the tobacco hornworm by 28% [112]. More recently, Fang *et al* (2014) found that engineering *M. acridum* to express a combination of four insect-specific toxins produced a synergistic effect and overall reduced the LT50 for acridids by 48% in comparison to the wild-type strain. When the engineered strains of *M. acridum* and the wild type were challenged with non-locust hosts (e.g., cockroach, mosquito), all were unable to cause infection and death [113]. This example of host specificity is of the utmost importance when introducing a biopesticide into the environment. The

careful evaluation of genetically engineered mycoinsecticides is required to prevent the accidental death of non-target organisms, thus *M. acridum* was a prime candidate for engineered virulence, as its host range is more specific when compared to some other *Metarhizium* species (e.g., *M. robertsii*) [54]. Host specificity has been an extremely important factor in mycoinsecticide research, and species of IPF with narrow host specificity are excellent candidates to overcome time-lapse problems associated with biological control [114].

Studies have also reported the involvement of chitin deacetylase in cuticle softening during pathogenesis and suggest its potential use in mycopesticide formulation to accelerate the eradication of insect pests from agricultural fields. Chitin deacetylase converts cuticular chitin to the easily degradable glucosamine polymer chitosan and thus facilitates easier penetration of the cuticle during insect pathogenesis [115]. Genetic manipulations of *Beauveria* and *Metarhizium* have shown improved virulence against numerous insect hosts. Improved cuticle penetration was observed in *B. bassiana* strains that express engineered proteases and chitinases [116, 117]. For example, chitin degradation was increased by a hybrid chitinase constructed by fusing a chitinase from *B. bassiana* with a chitin-binding domain from the silkworm [118].

Despite their positive influence on the environment, biocontrol agents are often slow acting in comparison to chemical applications [119]. The commercially available biopesticide, Green Muscle employs *M. acridum* to control locust and grasshopper populations; however, this treatment can take between one and three weeks to kill adult pests [120], and during this time, crop loss can be substantial. It is for this reason that the majority of myco-control research has been focused on improving the virulence of fungal pathogens [112, 113, 121, 122]. Furthermore, environmental factors, such as UV radiation, temperature, and humidity, affect the population of fungi in natural habitats, which in turn limits their utility as biocontrol agents [123].

Research could also focus on the identification of secondary metabolites involved in different stages of the fungal life cycle that can be utilized for strain improvement with increased stress tolerance [124]. LC-MS analysis of *Metarhizium* conidial extraction showed higher amounts of mannitol and a novel secondary metabolite, tyrosine betaine [124]. Mannitol is the main storage carbohydrate present in most fungi and is involved in improving oxidative and temperature stress tolerance in *Aspergillus nidulans* [123]. Tyrosine betaine is conserved in *Metarhizium* spp. and hence is speculated to be important for their biology; however, more analysis is needed to confirm this [124]. Ortiz-Urquiza and Keyhani [47] discussed the major signaling pathways involved in stress responses in both *Metarhizium* and *Beauveria*, thus providing insights into future directions that can be pursued for strain improvement. More research is needed toward effective pest management tools by developing persistent fungal strains with improved virulence and host range for field applications.

The endophytic capabilities of these EIPF have great potential in terms of biocontrol of soil-dwelling pests. For example, the redheaded cockchafer larvae, *Adoryphorus couloni*, is a common pasture pest in South Eastern Australia with subterranean feeding habits. This feeding habit of the redheaded cockchafer larvae renders aboveground-applied insecticides useless. Application of *Metarhizium* in the soil reduced the infestation of the larvae and increased pasture productivity [125, 126]. EIPF can live endophytically without causing any negative effects on plants, and plant colonization has been established by applying fungal propagules on either seeds or roots, thereby, providing opportunities to control soil-borne insects that cannot be easily controlled by chemical insecticides.

2.6.2 Plant protection and improvement

A fungal endophyte is defined as the occurrence of a fungal species living asymptomatically within the tissues of a plant [127]. The recent identification and investigation of IPF living as endophytes in various plants has revealed that in many cases this association is not just asymptomatic, but beneficial. A summary of the impacts and benefits conferred to plants via EIPF is shown in Figure. 2.1. The benefits conferred to a plant host depends on the fungal species involved and can be a single benefit or a combination of several benefits, including increase in plant biomass and productivity, alleviation of abiotic stresses (e.g., drought, salinity, temperature fluctuations) and improved resistance to biotic stress (e.g., herbivory and fungal disease). Vega (2008) and Quesada-Moraga *et al* (2014) provide comprehensive summaries of EIPF and plant hosts found to occur naturally, as well as successful symbioses obtained through artificial inoculation. *Metarhizium*, *Beauveria*, and *Isaria* are the most commonly isolated EIPF from temperate soils [84, 128]. In many instances, increased growth of the host plant occurs directly as a result of acquisition of growth-limiting nutrients (i.e., nitrogen and phosphorus) in exchange for plant-derived carbohydrates [129] or indirectly through alleviation of stress that would otherwise limit growth potential.

Recent studies with *Beauveria* and *Metarhizium* have demonstrated plant health promoting properties [25] and the important roles of these fungi in the ecological cycling of insect-derived nitrogen to plant communities [6, 89]. *Metarhizium* has been shown to increase plant biomass and mitigate salt stress in soybean in comparison to plants lacking fungal colonization [82]. Tomato, haricot bean, switchgrass, and soybean plants had increased root lengths and shoot/root dry weights [6, 25, 130] when colonized by various species of *Metarhizium*. The grass endophyte *Neotyphodium* has been shown to prevent defoliation and increase seed dispersal and vegetative

yield in *Bromus auleticus* [131]; it also protected *Bromus setifolius* from leaf-cutting ants [132]. EIPF are capable of successfully protecting plants from microbial pathogens by suppressing disease-causing agents or increasing plant defense responses. The fungal disease powdery mildew (*Sphaerotheca*) was suppressed by species of *Lecanicillium* (formerly *Verticillium*) and *Isaria* in cucumber and strawberry plants [133, 134], and *Metarhizium* was shown to be antagonistic toward the root-rot fungus, *Fusarium solani* [5]. *Beauveria* and *Lecanicillium* have been shown to induce the expression and production of plant defense compounds in the date palm, *Phoenix dactylifera* [135] and may contribute to plant "priming" against plant pathogens and increased plant growth [2], as seen with the endophytic mycoparasite Trichoderma [136]. *Lecanicillium* is also epiphytic and may prevent fungal disease by competitively growing on the surface of leaves, thus instilling spatial restrictions, producing antimicrobial compounds, limiting available nutrients, and being mycoparasitic, in addition to inducing plant responses while colonizing plant roots [2, 128]. With the knowledge that many EIPF have broad host ranges, in respect to both insect and plant hosts (e.g., *Metarhizium*, *Beauveria*, *Lecanicillium*), research should focus on establishing a method by which to measure the strength of symbiosis between certain species in order to ascertain the best candidate for pest control in a particular plant population.

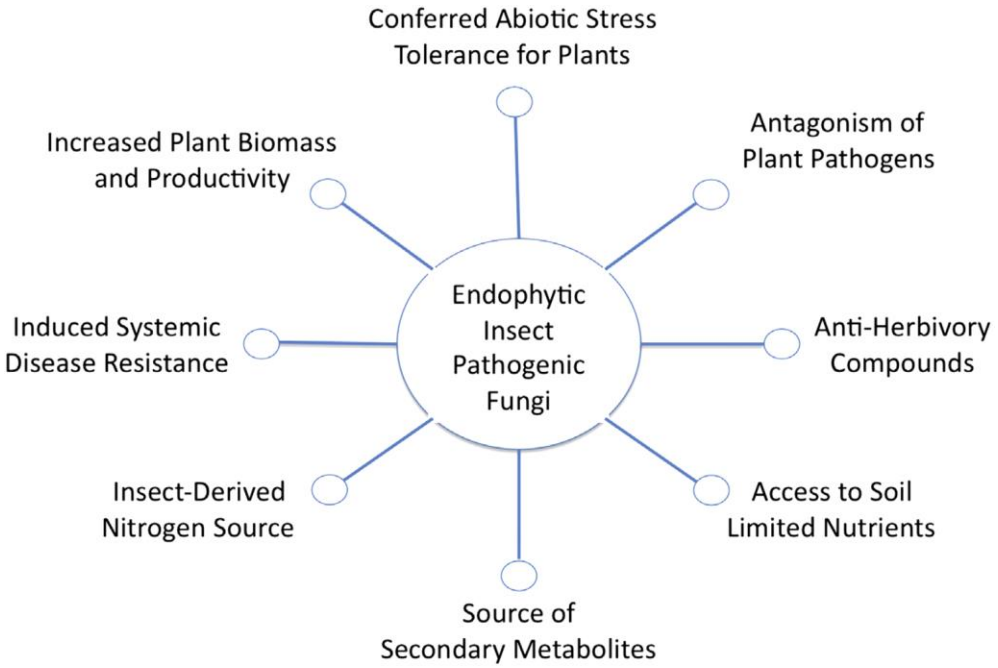

Figure 2.1. The applications, benefits, and impacts of endophytic insect pathogenic fungi colonization of plants.

2.7 Secondary metabolites

Aside from their role as biocontrol agents and as plant growth promoters, insect pathogens are rich sources of natural products/secondary metabolites, as well. Many gene clusters involved in secondary metabolite production have been predicted in IPF [137]. Analysis of secondary metabolite gene clusters in *Metarhizium* strains indicated that there are 85 and 57 core genes present in *M. robertsii* and *M. acridum*, respectively [138]. A variety of pharmaceutically relevant and insecticidal metabolites have been reported to date in *Metarhizium* including destruxins, fusarin-like compounds (NG39x), helvolic acid, cytochalasin, swainsonine, serinocyclins, viridoxins, and valicin [139–145]. Genome analysis predicts that the *B. bassiana* genome contains 45 core genes putatively involved in secondary metabolite synthesis [138] with the best-studied

secondary metabolites in *Beauveria* being beauvericin [146] and bassianolide [147]. Beauvericin has shown to have antimicrobial, insecticidal, and cytotoxic activities [146]. Bassianolide, also produced by other fungal species including *Lecanicillium*, has insecticidal and pharmacological properties [138].

In addition to the potential for secondary metabolite biosynthesis, these insect pathogens, specifically *Beauveria*, can be utilized as biocatalytic units for the transformation of chemical substrates [148]. In biotransformation, the enzymatic repertoire of the fungus is exploited for the transformation or modification of chemical compounds. *Beauveria* spp. are the most frequently used insect pathogens as biocatalysts for the transformation of chemicals. The primary reactions catalyzed by *Beauveria* are demethylation, hydroxylation, and glycosidations [138]. Although a number of secondary gene clusters have been predicted in these insect pathogens, the natural products emanating from these gene clusters remain to be identified.

Fungal endophytes are capable of synthesizing natural products/secondary metabolites when associated with plants, which have huge pharmaceutical potential. A well-studied example is the anticancer drug, Taxol produced by endophytic fungi when associated with yew trees belonging to the *Taxus* family [149]. *Metarhizium anisopliae* produces one of the highest yields of Taxol so far reported from an endophyte, 0.85 mg/L of fermentation broth (H-27 Accession #FJ375161) [137]. A more thorough understanding of the natural products produced during endophytic association by these fungi will contribute to the broader use of EIPF beyond their agricultural applications.

Chapter 3 - Hydrophobins contribute to root colonization and stress responses in the endophytic insect pathogenic fungus *Beauveria bassiana*

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3.1 Abstract

The hyd1/hyd2 hydrophobins are important constituents of the conidial cell wall of the insect pathogenic fungus, *Beauveria bassiana*. This fungus is also an endophyte of several plant species. Inactivation of two Class I hydrophobin genes, *hyd1* or *hyd2,* decreased the interaction of *B. bassiana* with bean roots. The Δ*hyd1/*Δ*hyd2* double mutant was less impaired in root association than *Δhyd1* or *Δhyd2*. Loss of *hyd* genes affected growth rate, conidiation ability and oosporein production. Expression patterns for genes involved in conidiation, cell wall integrity, insect virulence, signal transduction, adhesion, hydrophobicity and oosporein production were screened in the deletion mutants grown under different conditions. Repression of the major MAP-Kinase signal transduction pathways (*Slt2* MAPK pathway) was observed and was more pronounced in the single versus double *hyd* mutants under certain conditions. The Δ*hyd1/*Δ*hyd2* double mutant showed up-regulation of the *Hog1* MAPK and the *Msn2* transcription factor, under certain conditions, when compared to the wild type or single *hyd* mutants. Expression of the *bad2* adhesin and the oosporein polyketide synthase 1 gene was severely reduced in all mutants. In comparison, fewer changes were observed in the expression of key conidiation and cell wall integrity genes in *hyd* mutants compared to wild type. Taken together, the data from this study indicated pleiotropic consequences of *hyd1* and *hyd2* deletion on signaling and stress pathways, as well as the ability of the fungus to form stable associations with plant roots.

3.2 Introduction

Fungi capable of killing insects are recognized as biological control agents and several commercial products based on these organisms are currently available worldwide [15, 150]. More recently, several fungal species that are categorized as entomopathogens have also been isolated as epi- and endophytes from a wide variety of different plants which are termed endophytic insect pathogenic fungi (EIPF) [2]. EIPF are mainly Ascomycetes and have been reported in various genera including, *Acremonium*, *Metarhizium*, *Beauveria*, *Isaria*, *Cladosporium*, *Lecanicillium*, and *Trichoderma* [25, 78]. These findings have opened up new avenues for research and application, particularly the use of these fungi as plant growth promoters, beneficial rhizosphere colonizers and/or as antagonists to plant pathogens. In order to utilize these fungi in an agricultural setting, it is important to know the specific aspects of interaction of EIPF with their plant hosts.

For EIPF, the initial process of adhesion of fungal conidia to insect or plant host surfaces is critical for the successful maintenance of pathogenic (to insects) and/or mutualistic (to plants) relationships [3, 151]. Attachment of pathogenic or symbiotic fungal cells to different biological surfaces often involves both specific and non-specific interactions, either of which may precede the other [152]. Specific interactions are mediated by receptor-ligand-like interactions and are generally reversible, occurring either via binding of adhesion moieties within the fungal cell wall to receptors on the host cell surface, or vice versa, via fungal receptors that bind to host-cell surface features. Cell surface features, e.g. ionic charge and hydrophobicity, which can be affected by specific proteins, mediate non-specific interactions. Specific adhesion-protein-mediated interactions have been demonstrated in *Metarhizium spp*. that express two specific adhesins, Mad1 and Mad2 [37, 153]. Loss of *Mad2* via targeted gene inactivation, resulted in loss of binding to plant surfaces and decreased ability of the fungus to form rhizosphere interactions with plant roots

and *Mad1* deletion impaired binding of conidia to the insect surface and displayed reduced virulence [37]. Non-specific interactions can be mediated by various cell surface epitopes including carbohydrates and lipids as well as by proteins. Hydrophobins are amphipathic proteins that can self-assemble at water-air interfaces, and are unique to fungi [7, 154]. These proteins constitute what is known as the "rodlet layer" on fungal spores, due to the characteristic morphology these proteins impart on the fungal cell surface [155, 156]. The importance of hydrophobins during plant pathogenesis has been reported in several fungal species including *Magnaporthe oryzae* and *Clonostachys rosea* [157–160].

Beauveria bassiana is an EIPF in which both insect pathogenic and plant mutualistic (endophytic) mechanisms can be examined [4, 47, 161]. The availability of genetic and genomic resources has led to the characterization of pathways involved in cuticle degradation and assimilation, secondary metabolite production and regulation, signal transduction and stress responses [138, 162]. However, while the biochemical and genetic mechanisms of insect pathogenesis by *Beauveria* is relatively well understood [163], little is known of the molecular determinants mediating plant interactions. Previous studies have shown that the initial adhesion of *B. bassiana* during insect pathogenesis is facilitated by non-specific hydrophobic interactions mediated, in part, by hydrophobins; although other factors have also been characterized. Two hydrophobin genes, *hyd1* and *hyd2*, which encode for Class I hydrophobins, have been shown to differentially affect fungal cell surface features and virulence. Loss of *hyd1* disrupted rodlet layer formation, decreased spore hydrophobicity, and impaired fungal infection of insects. In contrast, more minor effects were seen for Δ*hyd2* mutants, with little to no effect on insect virulence. The double Δ*hyd1*/Δ*hyd2* mutant showed additive effects with a dramatic reduction in virulence [10].

Here we investigated the involvement of *hyd1* and *hyd2* in root colonization using haricot bean, *Phaseolus vulgaris*, as a model system. Root colonization assays revealed differential impairment of the Δ*hyd1*, Δ*hyd2*, and Δ*hyd1*/Δ*hyd2* mutant in their ability to form plant associations. Unexpectedly, the Δ*hyd1*/Δ*hyd2* double mutant was less severely impacted in these assays than the single mutants. Stress and growth response profiling indicated differential effects on growth, conidiation, and expression profiles of genes involved in signal transduction and adhesion. These data indicated a role for hydrophobins in plant association, coupled with the complex pleiotropic consequences of the loss of the hydrophobins on signaling pathways.

3.3 Materials and Methods

3.3.1 Growth and maintenance of fungal cultures

Beauveria bassiana (ATCC 90517*)* wild type (WT), and the Δ*hyd1*, Δ*hyd2*, Δ*hyd1*/Δ*hyd2,* Δ*hyd1:hyd1 and* Δ*hyd2:hyd2* strains (Table 3.1) have been previously reported [10]. Fungal cultures were routinely grown and maintained on Sabouraud dextrose, Czapek-dox, or Potato dextrose agars (SDA, CZA, and PDA, Bioshop Canada Inc., Burlington, ON) as needed. Plates were grown at 27^{o}C, with PDA routinely used for harvesting of conidia after 10-14 days (d) of growth followed by collecting conidia after flooding of the plates with sterile 0.01% Triton X-100. Conidia were counted using a haemocytometer and adjusted to specific concentrations (typically between 10^6-10^7 conidia/mL) as needed.

Table 3.1 Fungal strains used in this study

Fungal Isolate	Selection marker	Genotype	Reference
B. bassiana ATCC 90517	-	Wild type (WT)	[10]
B. bassiana Δ*hyd1*	bar*	Δ*hyd1* mutant	
B. bassiana Δ*hyd2*	bar	Δ*hyd2* mutant	
B. bassiana Δ*hyd1*/Δ*hyd1*	bar, sur**	Δ*hyd1*/Δ*hyd2* double mutant	
B. bassiana Δ*hyd1:hyd1*	bar, sur	Δ*hyd1:hyd1* complemented	
B. bassiana Δ*hyd2:hyd2*	bar, sur	Δ*hyd2:hyd2* complemented	

* Bar selective marker confers resistance to phosphinothricin.
** Sur selective marker confers sulfonylurea resistance.

3.3.2 Phenotypic analysis

Fungal vegetative growth rates and conidiation/conidial yields of the WT and hydrophobin (*hyd*) mutants were assessed on PDA and YPDA (yeast extract-peptone-dextrose agar) (Bioshop Canada Inc., Burlington, ON) in petri dishes containing 10 mL of media. Five µL of fungal conidial suspensions ($1x10^7$ conidia/mL in 0.01% TritonX-100) were inoculated onto the centre of the agar plate and incubated at 27°C. Radial growth rates were recorded by measuring colony diameters at 3, 7 and 14 days post-inoculation. Conidial yield was quantified by harvesting conidia from 14 day old PDA plates for each isolate. Briefly, a 0.8 mm diameter agar plug was collected from the centre of the colony and the plugs were vortexed for 2 minutes in 0.01% Triton X-100. Conidia were microscopically counted using a haemocytometer. Five replicates were prepared for each isolate and the experiment was repeated twice with independent batches of conidia.

Pigmentation or oosporein production was assessed by inoculation of fungal conidia ($1 x 10^7$ conidia/mL) into 15-mL YPD broth and grown with agitation (120 rpm) at 27°C for 4 days.

Conidial germination was measured on PDA plates as follows; 50 µL aliquots of a $1x10^4$ conidia/mL suspension were spread on PDA plates and incubated at 27ºC. The percentage of germinated conidia was measured by microscopic analysis of at least 100 conidia/replicate. A conidium was considered germinated when the germ tube was at least the length of the diameter of the conidial cell (>3-4 µm). Five replicates were prepared for each fungal isolate.

3.3.3 Bioconversion analysis

Orsellinic acid (OA) (Thermo Fisher Scientific, Waltham, MA) was added to YPD broth at four different concentrations (10mg/mL, 1mg/mL, 0.1mg/mL and 0.01mg/mL) as a precursor molecule in order to analyze oosporein production in WT and *hyd* mutants. Aliquots (100 µL) of conidial suspensions ($1x10^6$ conidia/mL) of WT and mutant strains were inoculated into 100 mL of YPD broth containing OA and cultures were incubated at 27ºC for 4 days with agitation (120 rpm).

3.3.4 Penetrant germ tube formation and production of ROS

Penetrant germ tube formation was assayed by inoculating 10 µL of $1x10^5$ conidia/mL onto peeled onion epidermis. After 30 hours, the onion epidermis was stained with lactophenol cotton blue and germ tube formation was observed under microscope. The generation of reactive oxygen species (ROS) was visualized on mutant strains using Nitro blue tetrazolium (NBT) assay as previously reported [164]. The *B. bassiana* WT and *hyd* mutant strains were grown on Sabouraud dextrose broth for at 27ºC. The mycelia were harvested after 36 hours and were incubated for 20 minutes in 0.3mM NBT aqueous solution containing 0.3mM NADPH. NBT on reduction by ROS, formed purple precipitate indicates the production of ROS.

3.3.5 Root colonization assays: CFU and semi-quantitative PCR

Root colonization assays were performed using *Phaseolus vulgaris* (haricot bean, cultivar soldier) obtained from OSC Seeds (Kitchener, ON). The seeds were surface sterilized with 4% sodium hypochlorite solution (NaOCl) three times for 5 minutes. The seeds were rinsed with sterile distilled H_2O after each NaOCl wash. Seeds were then soaked in 15% hydrogen peroxide (H_2O_2) for 5 minutes and subsequently rinsed five times with sterile distilled H_2O to remove residual hydrogen peroxide. Seeds were then kept overnight at 4^oC for synchronization of growth before planting and fungal inoculation. Seeds were placed in sterile vermiculite and kept at 25^oC for a photoperiod of 16 hours per day for 3-4 days until germination. The 3-4 d old germinated seedlings were then planted in pots containing sterile vermiculite (Ther-O-Rock East Inc., New Eagle, PA). Five replicates of each treatment were individually planted in each pot for each fungal strain tested. Conidial suspensions for the WT and *hyd* mutants were prepared from 14 day PDA plates as described above. Fungal inoculation was performed using a soil drench method with modification to the conidial concentration [165]. Briefly, conidia were harvested in 0.01% Triton X-100 and the concentration of the conidial suspensions were adjusted to $1x10^7$ conidia/mL. Aliquots (5 mL) of the conidial suspensions were evenly poured over the vermiculite surface of each pot. Control bean seedlings were treated with sterile Triton X-100. All pots were kept in a greenhouse at 25^oC during the day and 18^oC during the night with photoperiod of 16:8 h light:dark cycle and a relative humidity between 62-80%. Plants were watered daily with sterile distilled H_2O. Bean roots were collected from 3 and 7 day old plants and then washed with sterile H_2O to remove the vermiculite attached to the roots. The roots were weighed and cut into ~0.2-0.5 mm pieces and homogenized (Biospec Products Inc., Bartlesville, OK) for 2 minutes [94]. The root homogenate were assayed for fungal recovery of colony forming units (CFU) on modified CTC agar [166] (YPDA

supplemented with 0.5 g/L chloramphenicol, 0.004 g/L thiabendazole and 0.5 g/L cycloheximide) and CFU values were calculated as CFU/100 mg of root weight.

Fungal colonization on roots was also quantified using semi-quantitative PCR. Samples of the bean roots harvested at 7 days post-inoculation were washed, weighed and then ground in liquid nitrogen using a mortar and pestle. Total DNA was extracted using CTAB (cetyl trimethylammonium bromide) method. The plant roots after harvest was washed in sterile distilled water and fresh root weights were recorded. The roots were then ground in liquid nitrogen using a mortar and pestle. Five volumes of preheated (65^{o}C) CTAB buffer were added to every 1 volume (1g) of root. Samples were then incubated at 65^{o}C for 30 minutes and subsequently treated with RNaseA (Thermo Fisher Scientific, Waltham, MA). The supernatant was collected by centrifugation and phase separated in chloroform:isoamyl alcohol. The DNA pellet was collected after precipitating each sample with isopropanol. Finally, the DNA pellet was washed in 70% ethanol and nuclease free water was then added to each sample after air drying. The concentration of the DNA sample was adjusted to 10 ng/µL and used as the template for PCR (MyiQ, Bio-Rad, Hercules, CA). DNA extracted from the WT strain was used to construct a standard with concentrations ranging from 10 ng to 0.1 ng for quantification of the DNA present in the experimental samples. The primers used for quantification were Bb ITSF (5'GAACCTACCTATYGTTGCTTC) and Bb ITSR (5'ATYCGAGGTCAACGTTCAG) [167].

3.3.6 Gene expression analysis and semi-quantitative RT-PCR

B. bassiana WT and the *hyd* mutants were grown on PDA for 14 days and conidia were collected from the agar plates using 0.01% Triton X-100. Conidial concentrations were adjusted to 1x10^{7} conidia/mL, and 2 mL of the conidial suspension was used to inoculate 200 mL of YPD broth. Cultures were then incubated with agitation (120 rpm) at 27^{o}C. Fungal mycelia were

collected after 4 days by vacuum filtration and were equally divided (~0.25 g of mycelia) and used as the inoculum into separate flasks containing 15 mL of medium. Eleven different culture growth conditions were examined; (1) distilled water, (2-4) three concentrations (v/v) of bean root exudate (1%, 10%, 100%), (5) 1% (w/v) chitin, (6) 1% (w/v) tomato stem, (7) 1% (w/v) glucose, (8) 1% (w/v) trehalose, (9) 1% (w/v) raffinose, (10) 1% (w/v) cellulose and (11) YPD. Cultures were then incubated with agitation (120 rpm) at 27ºC and the fungal mycelia were harvested by vacuum filtration 12 hours after inoculation. Cultures corresponding to an additional three stress conditions (with a H_2O control) were inoculated as above, but harvested after 6 hours of incubation. These conditions included cultures supplemented with (a) 300 µg/mL Congo red, (b) 20 mM H_2O_2 and (c) 500 mM NaCl.

Bean root exudate (BRE) was prepared after bean seeds were sterilized as described above. The sterile seeds were then germinated on water agar (1%) for 4 days and then transferred to a flask containing sterile distilled H_2O. Approximately 25 seedlings were used for 500 mL of sterile distilled H_2O. Seedling cultures were incubated at room temperature with agitation (100 rpm). The bean root exudate was collected after 4 days and the sterility of the exudate were confirmed by plating aliquots onto PDA plates.

The fungal mycelia from each growth condition were harvested by vacuum filtration and the total RNA from each sample was extracted using TRI- reagent (Sigma Aldrich Canada Ltd., Oakville, ON) [153]. The extracted RNA was then treated with RQ1 RNase-free DNase (Promega, Madison, WI) and the RNA concentration was determined spectrophotometrically using Qubit (Invitrogen, Carlsbad, CA). cDNA for each sample was generated using the High Capacity cDNA reverse transcription kit (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA) with 4 µg of total RNA in a total volume of 40 µL, following manufacturer's instructions. The transcript

levels of several genes (Supporting Information, Table S3.1) were assessed via semi-quantitative PCR (My iQ, Bio-Rad, Hercules, CA) using 2X PCR master mix (Norgen Biotek Corp., Thorold, ON), and the 18S rRNA as the reference gene. RT-PCR reaction mixtures contained 10 µL 2X PCR master mix, 2 µL cDNA, and 1 µL of each of forward and reverse primers (2.5 µM) and made the reaction to 20 µL with nuclease-free water. PCR cycling conditions were as follows: 95ºC (2 min), 30 cycles of 95ºC (30 s), 52ºC-60ºC (45 s), 72ºC (1 min), and final extension at 72ºC (3 min). Aliquots (5 µL) of the RT-PCR reaction product were run on 2% agarose gel electrophoresis (30 min at 80V) for quantitation. Images of the GelRed (Biotium Inc., Fremont, CA) stained agarose gels were acquired with Gel Doc EZ Imaging System (Bio-Rad, Hercules, CA). The quantification of intensity of bands was performed using Image Lab (Bio-Rad, Hercules, CA) software. The ratio of band intensity of the corresponding gene of interest to the band intensity of reference gene (18s) was calculated to normalize the variations in concentrations in each sample. The relative expression was quantified after normalization and the mean standard error of for all conditions were calculated [168]. Experiments were performed using three biological replicates and primer sequences used to evaluate gene expression patterns are shown in Table S3.1.

3.4 Results

3.4.1 *B. bassiana* hyd1 and hyd2 affect growth rate, conidiation and pigment production

Subtle differences in colony morphology were observed between the WT and the Δ*hyd1*, *Δhyd2*, and Δ*hyd1/*Δ*hyd2* mutants when grown on PDA (Figure 3.1a). All strains formed white/off-white colonies. The Δ*hyd2* and Δ*hyd1/*Δ*hyd2* mutants produced fluffier colonies than the WT or Δ*hyd1* strains. Disruption of hydrophobins, however, affected both growth and conidiation on PDA (Figure 3.1b & c). Decreased growth was observed for the *hyd* mutants at 14 days (ANOVA, p

<0.001). Conidial yield was also decreased (2-4 fold) for the *hyd* mutants compared to WT (p <0.01). The complemented mutant strains showed partial restoration of growth and complete restoration of conidiation. No differences in the conidial germination rates were found between the WT and mutant strains, with all reaching >90% germination after 24 h of growth (Figure 3.1d). In liquid culture, the loss of oosporein production (red pigment) was evident for the *hyd* mutants (Figure 3.1e). The loss of oosporein production could not be restored in *hyd* mutants even after adding orsellinic acid, the major precursor for oosporein production, to the growth media. No differences in penetrant germ tube formation were noticed between the WT, single and double *hyd* mutants (Figure 3.2) on onion epidermis. ROS production was examined using an *in situ* semi-quantitative reactive oxygen species dye-based assay (nitro blue tetrazolium) showed hyphae derived from the *hyd* mutants (Δ*hyd1*, Δ*hyd2*, and Δ*hyd1/*Δ*hyd2* strains) showed extensive blue staining particularly at hyphal tips, with little visible staining observed in the WT parental strain (Figure 3.3).

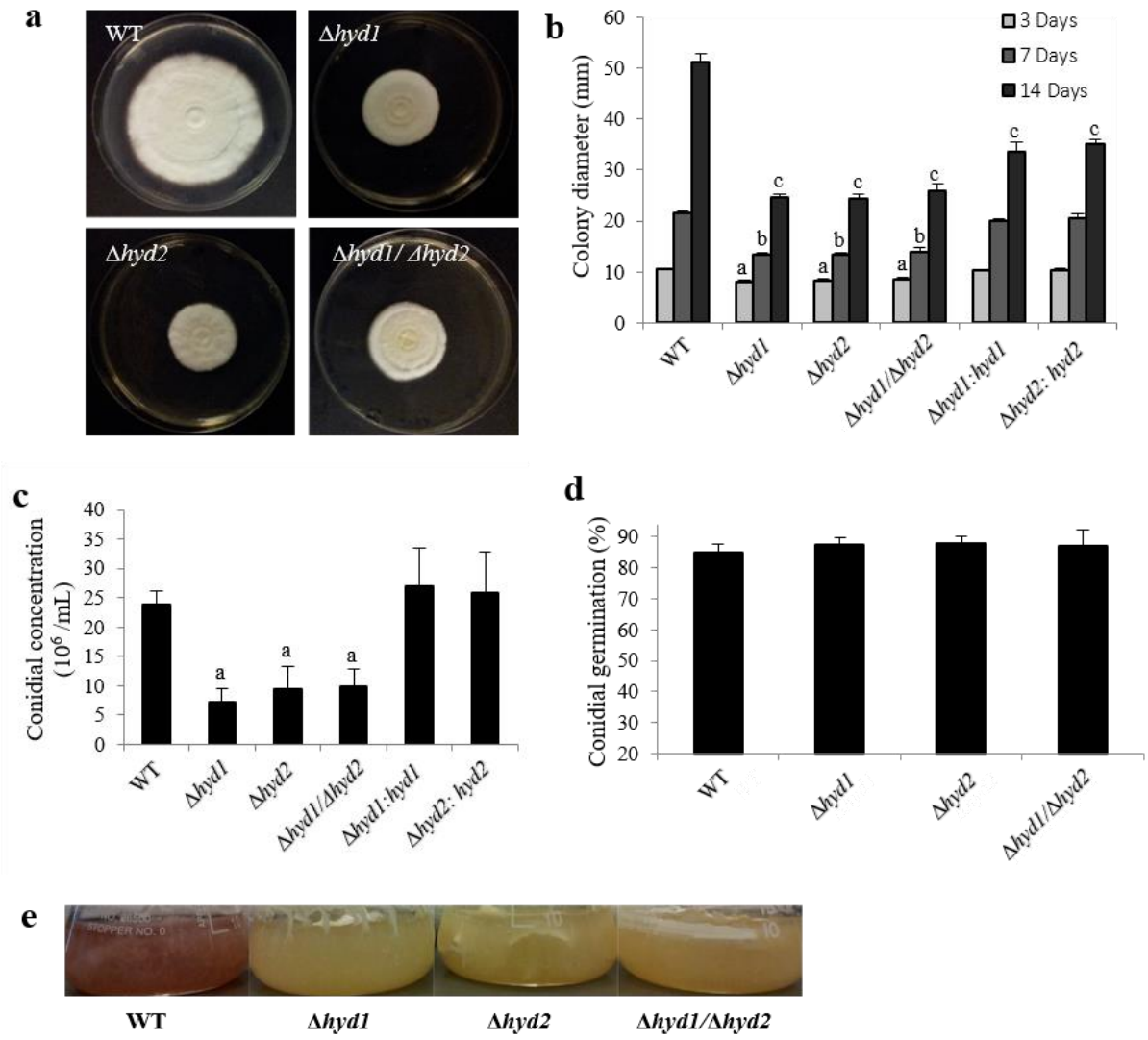


Figure 3.1 a. Colony morphology and radial growth rate of WT and *hyd* mutant strains on 14 day old PDA plate. The experiment was performed in five replicates and images of representative plates were taken 14 days post inoculation. **b. Growth rate.** Radial growth rate of *B. bassiana of* WT and *hyd* mutants in PDA after inoculation with 10 µL of 10^7 conidial/mL in the centre of the plate containing 10 mL of media, followed by incubation at 27ºC. Colony diameter was measured at 3, 7 and 14 days. Data are the mean ± standard deviation of five replicates. **c. Conidiation.** Quantification of conidial production of *B. bassiana* WT and *hyd* mutants of the fungal isolates on PDA medium. Four replicates were counted for each isolate. Error bars represent the standard deviation of the mean. **d. Conidial germination.** Conidial germination of *B. bassiana* WT and *hyd* mutants were measured on PDA media. The percentage of germinated conidia after 24 hours was recorded by microscopic examination of at least 100 conidia/replicate. Data shown are the mean ± standard deviation of five replicates**. e. Oosporein production of *B. bassiana* WT and *hyd* mutants in YPD broth**. Cultures were inoculated with 150 µL of 1x 10^6 conidial/mL in 15 mL of YPD broth and were grown in shake culture at 120 rpm at 27ºC for 4 days. The oosporein production can be observed in *B. bassiana* WT and not in mutant strains (Δ*hyd1*, Δ*hyd2*, and Δ*hyd1*/Δ*hyd2*). The letters in the graph denote significant differences relative to WT.

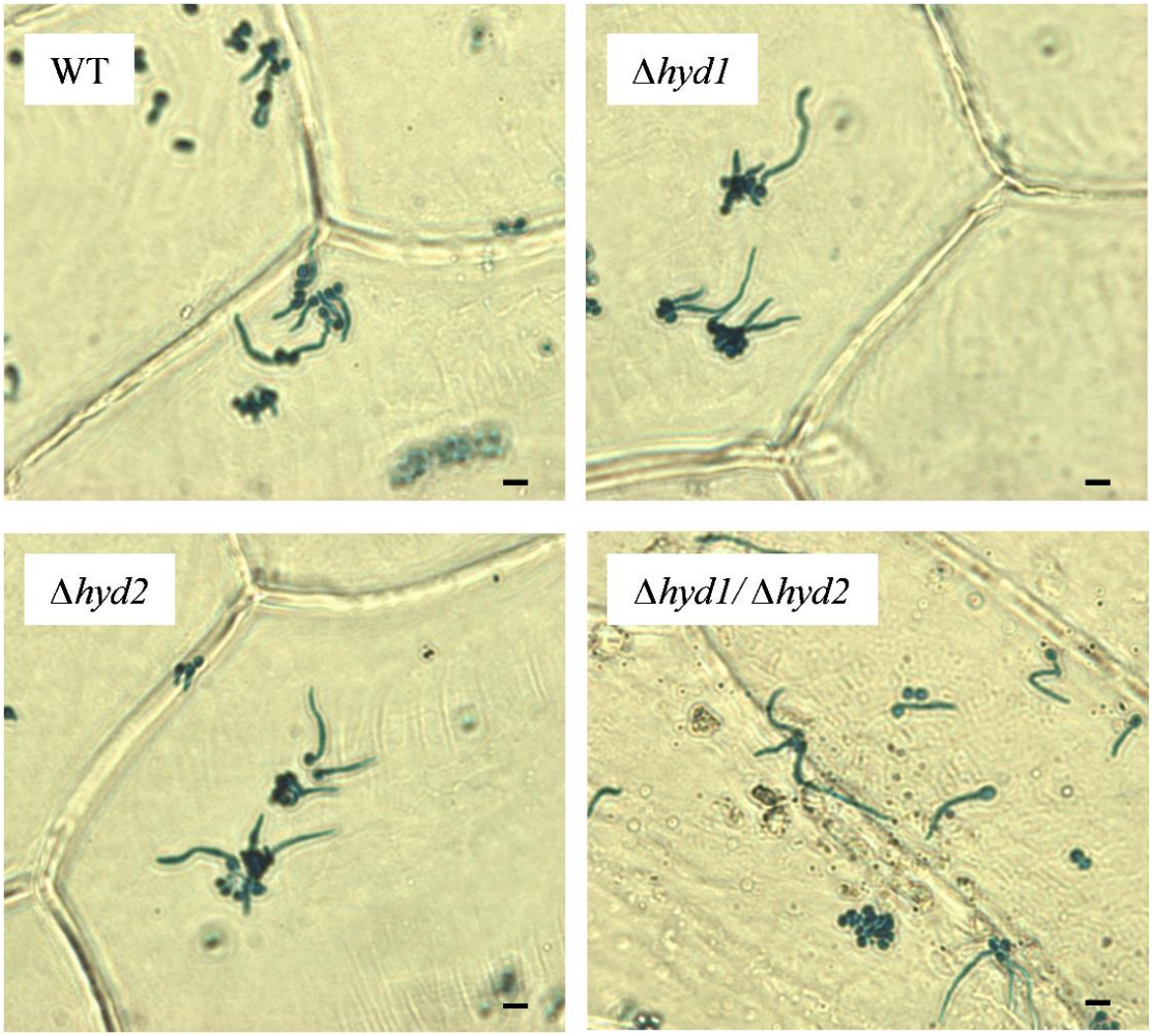


Figure 3.2 Penetrant germ tube formation of *B. bassiana* WT and *hyd* mutant strains on onion skin epidermis. Scale bar = 2.5 μm.

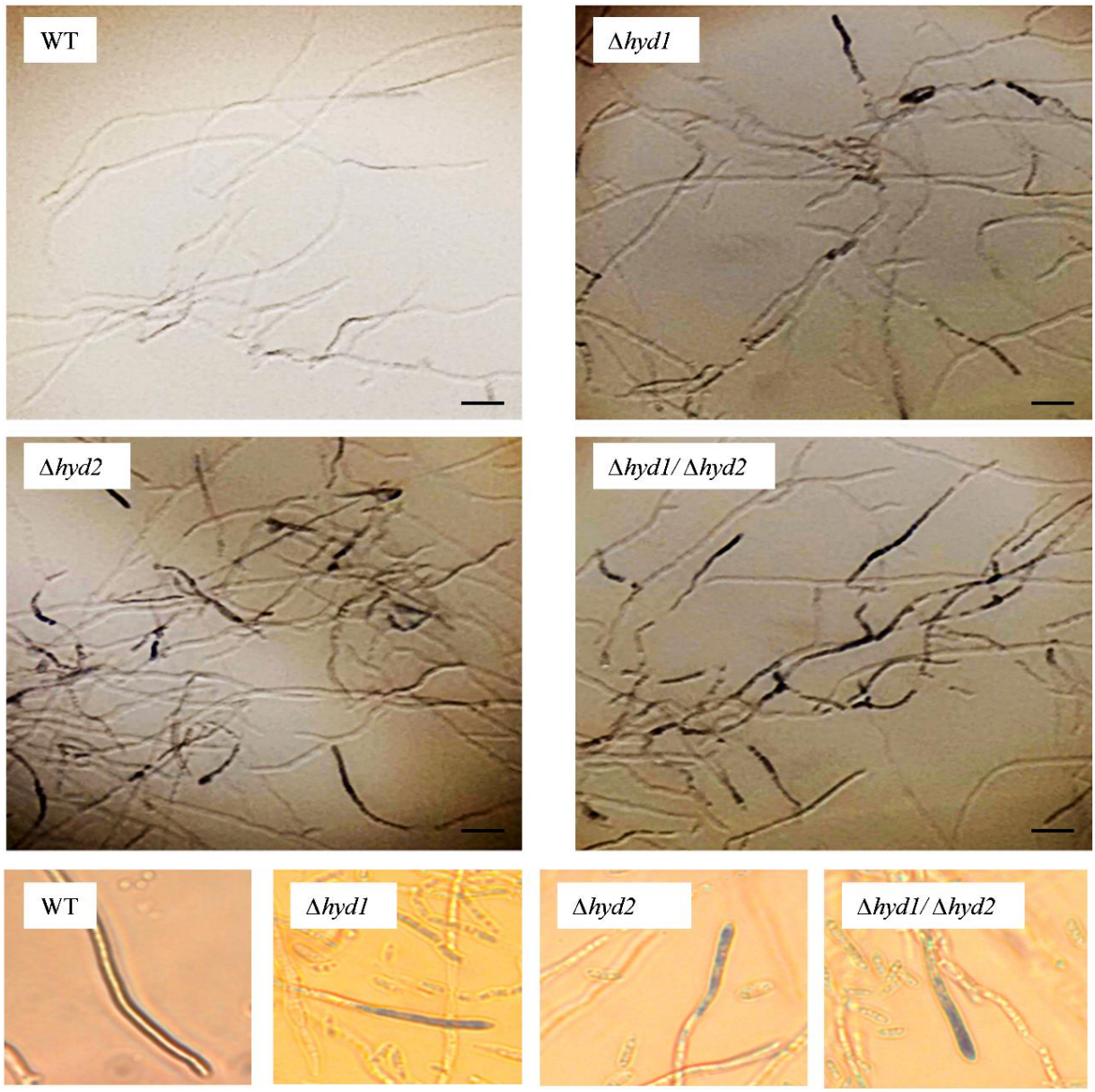


Figure 3.3 ROS production in *hyd* mutants. The *B. bassiana* WT and *hyd* mutant strains were grown on Sabouraud dextrose broth for at 27°C. The mycelia were harvested after 36 hours and were incubated for 20 minutes in 0.3mM NBT aqueous solution containing 0.3mM NADPH. NBT on reduction by ROS, formed purple precipitate indicates the production of ROS. The bottom panel shows the enlarged hyphal tip of Δ*hyd1,* Δ*hyd2* and Δ*hyd1/*Δ*hyd2* respectively showing purple/blue color.

3.4.2 Loss of *hyd1* and *hyd2* genes decreases the association of *B. bassiana* with bean roots

In order to test whether hydrophobins are important in mediating plant root interactions, haricot bean (*P. vulgaris*) root colonization assays were performed as detailed in the Methods section. Recovery of *B. bassiana* fungal colony forming units (CFUs) from the roots of treated plants revealed a decrease in plant root association abilities for the *hyd* mutants as compared to the WT (Figure 3.4a). For the WT, a high concentration (~20,000 CFUs/100 mg root) was seen 3 days post-inoculation on the soil that decreased by ~50% by 7 d post-treatment. In contrast, a dramatic decrease of recoverable CFUs was seen for both the Δ*hyd1* and Δ*hyd2* mutants at both 3 and 7 days post-treatment as compared to the WT; 2-way ANOVA, Sidak's multiple comparison test, WT v/s Δ*hyd1* ($p < 0.001$), WT v/s Δhyd2 ($p<0.001$), for both the 3 and 7 days time points. Recoverable CFUs for the Δ*hyd1* mutant was ~ 4000/100 mg root at 3 days and ~2500 CFU/100 mg root at 7 days, and even lower for the Δ*hyd2* mutant, ~2000 CFUs at 3 and 7 days. Surprisingly, the Δ*hyd1/*Δ*hyd2* mutant was not as impaired as the single *hyd* mutants in terms of plant root association. At 3 days post inoculation, ~10,000 CFUs/ 100 mg root was detected for the Δ*hyd1/*Δ*hyd2* strain (WT v/s Δ*hyd1/hyd2*, $p<0.007$). However, the Δ*hyd1/*Δ*hyd2* mutant showed similar levels of root plant association as the WT at 7 days. In order to examine whether the mutants had either persistence or some other issues with survival in the soil, CFU in vermiculite collected surrounding the plant roots (3 and 7 days) was determined (Figure 3.4b). Similar levels of CFUs were recovered from the WT and mutants at 3 and 7 days, with the exception of higher numbers for the Δ*hyd2* mutant strain in the vermiculite at 3 days (WT v/s Δ*hyd2* ($p<0.003$)). Plant root association was also examined via semi-quantitative PCR amplification of DNA extracted fungus-root associated samples as detailed in the Methods section. These data were consistent with the CFU recovery results in which higher levels of fungal DNA were seen in WT–haricot bean

root samples, lower in both the Δ*hyd1* and Δ*hyd2* mutants, with greater fungal DNA content seen for the Δ*hyd1*/Δ*hyd2* mutant as compared to the single mutants, but still lower than WT (Figure 3.4c).

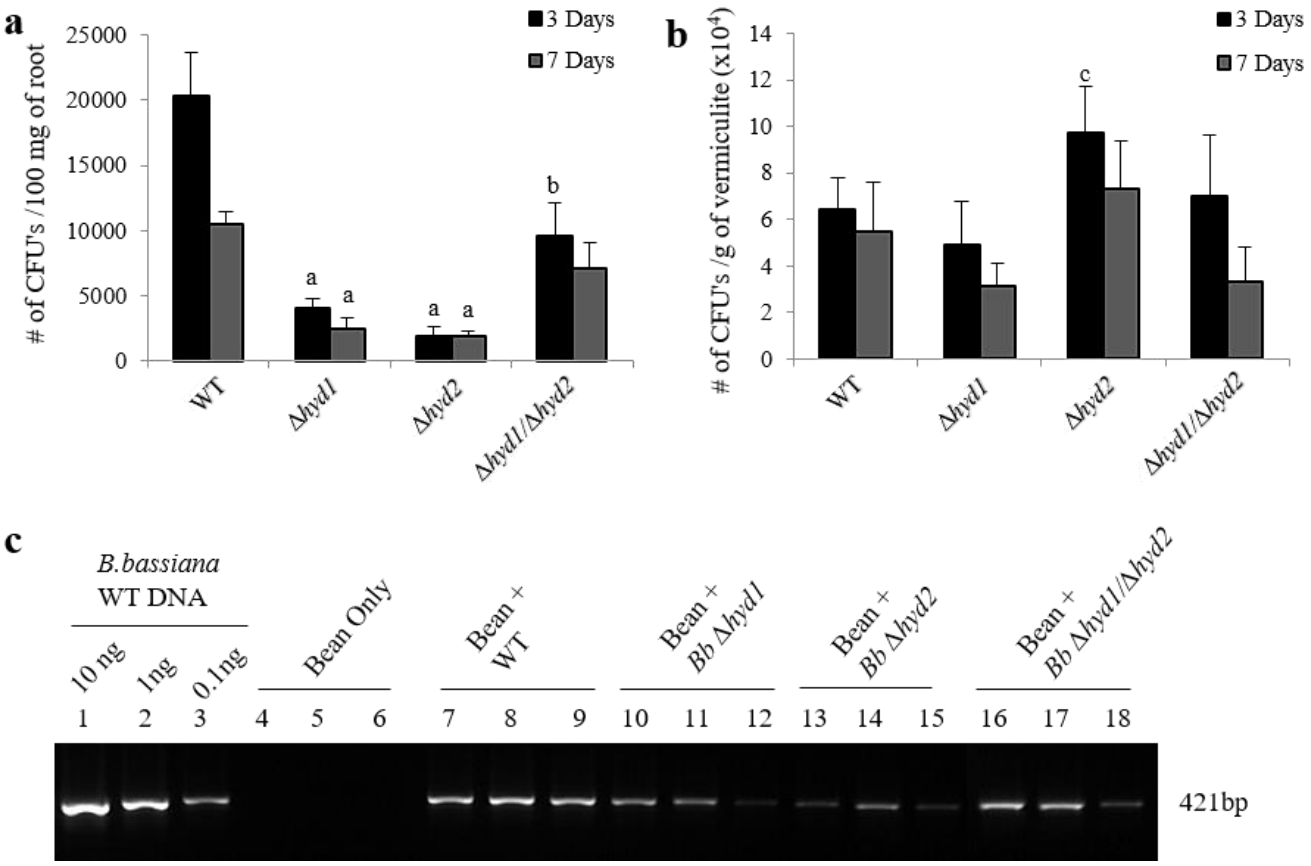


Figure 3.4 a. Haricot bean (cultivar-soldier) root colonization by *B. bassiana* WT and *hyd* mutants. 3- 4 days old germinated seedlings were planted on sterile vermiculite inoculated with 5 mL of 10^7 conidia mL^{-1}. Bean roots were harvested on 3 and 7 days post inoculation and washed in water. The roots were then homogenized and plated on selective media plates. The CFUs were counted after 7 days and CFUs/100g of root weight was calculated. **b. Rhizosphere association by *B. bassiana* WT and *hyd* mutants.** ~0.5±0.25 g of vermiculite surrounding root was collected on the day of 3 and 7 day harvest. Vermiculite was suspended in 0.01% Triton X-100 and 0.1 mL was plated on selective media after serial dilution. The CFU's were counted after 7 days and CFUs/g of soil weight was calculated. **c. Semi-quantitative PCR used to detect the presence of fungal in plant roots.** *B. bassiana* WT DNA with known concentration was used as the standard. The letters in the graph denote significant differences relative to WT.

3.4.3 Loss of *B. bassiana hyd* genes affects the expression of key signal transduction, adhesion and pigment production genes

Semi-quantitative RT-PCR was used to analyze the expression patterns of 20 genes implicated in virulence, stress response, and cellular signaling. These included genes involved in (a) conidiation; *FlbA*, *FlbB*, and *FlbD*, (b) insect virulence; *CDEP1* cuticle degrading protease, *Chi1* chitinase, *Chit2* endochitinase, and the *ChsA2* chitinase, (c) signal transduction; *ras1*, *ras2*, *ras3*, *Hog1* MAPK (*high-osmolarity glycerol 1* mitogen-activated protein kinase), *Slt2* MAPK (mitogen-activated protein kinase), *Mkk1* MAPKK (mitogen-activated protein kinase kinase), *Bck1* MAPKKK (mitogen-activated protein kinase kinase kinase), and the *Msn2* transcription factor, (d) adhesion/cell surface properties; *hyd1*, *hyd2*, *bad1* (*Beauveria* adhesin 1, homolog to the *Metarhizium* adhesin 1 implicated in binding to the insect cuticle), and *bad2* (*Beauveria* adhesin 2, homolog of the *M. robertsii* adhesin 2, implicated in binding to plant surfaces), and (e) secondary metabolite production; *Ops1*, oosporein polyketide synthase (*pks9*). Gene expression analysis was performed on RNA extracted from the mycelia of the WT and *hyd* mutant strains grown in YPD broth and then subsequently transferred to media indicated in the Methods section. The expression patterns of *FlbA, FlbB, FlbD, Chi1, Chi2, ChiA2, ras genes, hyd1* (except in the respective mutant strains), *hyd2* (except in respective mutant strains), and *bad1* were similar between the WT and mutant strains in all of the conditions examined (data not shown).

A general pattern in which expression of the signal transduction proteins *Slt2*, *Bck1* and *Mkk1* were more highly expressed in the WT > Δ*hyd1*/Δ*hyd2* mutant > *Δhyd1*, *Δhyd2* was seen when grown in water, bean root exudate (100%, 1%), 1% chitin, and 1% tomato stem extract. No differences in expression of *Slt2*, *Bck1* or *Mkk1* were found between the WT and mutant strains in different carbohydrates and under stress (Congo red, H_2O_2 and NaCl), with the exception of the higher *Mkk1* expression for Δ*hyd2*, Δ*hyd1*/Δ*hyd2* for hydrogen peroxide and NaCl (Figure 3.5 a-c

and Supporting Information - Figure S3.1 a-c). Interestingly, the expression of the *Hog1* MAPK was found to be consistently higher for the Δ*hyd1*/Δ*hyd2* mutant strain when compared to the WT and single mutant strains. Similarly, the expression of the *Msn2* transcription factor was higher in the Δ*hyd1*/Δ*hyd2* when grown in bean root exudate or 1% tomato stem. However, expression levels of *Msn2* were similar in the WT and mutant strains in 1% chitin, YPD, different carbohydrates and stress conditions (Congo red, H_2O_2 and NaCl) (Figure 3.5 a-c and Figure S3.1 d & e). Expression of the *bad2* adhesin as well as the *Ops1* polyketide synthase (*pks9*) was dramatically reduced in the *hyd* mutants (Figure 3.5 a-c and Figure S3.1 g and h). Transcript levels of *bad2* in WT versus *hyd* mutants revealed that the expression of *bad2* was decreased in the single and double mutant strains. However, the expression of *bad2* was found to be elevated in the mutant strains under stress (Congo red, H_2O_2 and NaCl) (Figure 3.5 a-c and Figure S3.1 g). The expression pattern of the cuticle degrading protease, *CDEP1*, was observed to be similar in the Δ*hyd1*, Δ*hyd1* and Δ*hyd1*/Δ*hyd2* isolates. However, *CDEP1* was upregulated in the WT strain when grown in the presence of BRE (100%, 10% and 1%) and water (6 hours) (Figure 3.5 a-c and Figure S3.1f).

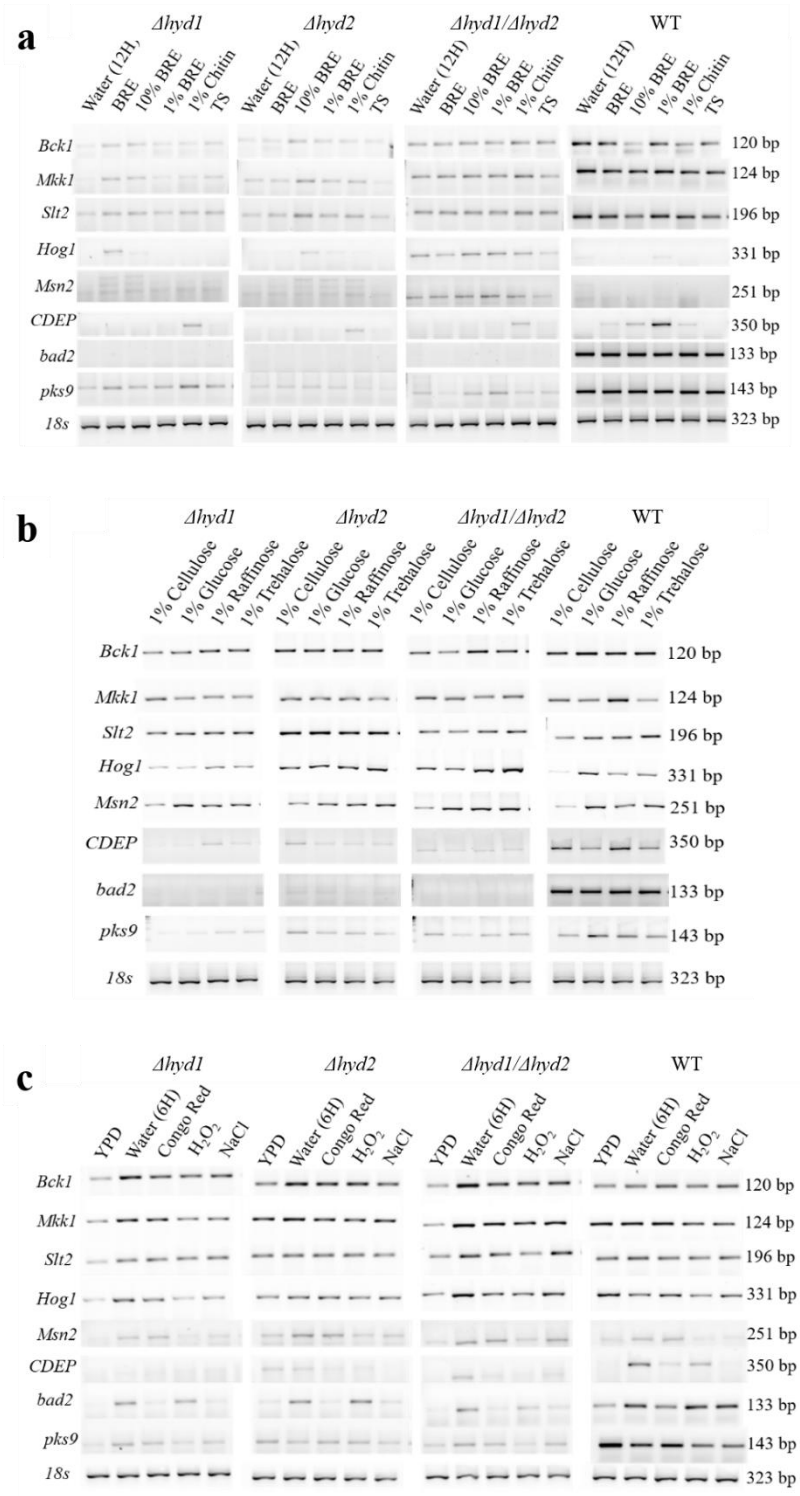


Figure 3.5 <u>Semi-quantitative RT PCR of selected genes under different conditions</u>. Relative intensity of selected genes for WT and mutant strains in, **a.** Water (12H), Bean root exudate (BRE - 100%, 10% and 1%), 1% chitin, 1% tomato stem extract (TS), **b.** Different carbohydrates in water (1% Cellulose, 1% Glucose, 1% Raffinose and 1% Trehalose), **c.** YPD and stress conditions (Congo red, H_2O_2 and NaCl). The image of agarose gel shown is the representative from 3 replicates studied for each condition.

3.5 Discussion

The insect pathogenic fungus *B. bassiana* is best known as an insect pathogen, however, more recently, their ability to form epiphytic or endophytic symbiotic associations with plants has been recognized [4]. Mutualistic interaction between plants and EIPF has been shown to include mobilization of nitrogen from the insect pathogenic activity of EIPF to the plant host, and secretion of plant carbohydrates utilizable by the fungus [6]. Hydrophobins contribute to the hydrophobic nature of many fungal spores or conidia, and in *B. bassiana*, two hydrophobins, *hyd1* and *hyd2* have been shown to differentially contribute to the rodlet layer and insect virulence [10]. However, any role(s) for *B. bassiana* hydrophobins in mediating plant associations have not been reported. Our data demonstrate that *hyd1* and *hyd2* contribute to the ability of the fungus to form root associations (in contrast *hyd2* was mainly implicated in insect virulence). Root colonization bioassays revealed that for the plant pathogenic fungus, *C. rosea*, a hydrophobin (*hyd3*) was similarly needed for colonization of *Arabidopsis thaliana* and barley roots [160]. In addition, *Tashyd1*, a Class I hydrophobin gene in *Trichoderma asperellum*, was found to contribute to root colonization. Deletion mutants of *Tashyd1* showed reduced colonization of plant roots when compared to the wild type [169]. However, our data indicated that the requirement/contribution of hydrophobins may be more complex than originally considered, as plant association was greater for the double Δ*hyd1*/Δ*hyd2* mutant than for each single mutant. This was not due to any generalized loss of survival in the soil since fungal recovery from the surrounding (to the root) vermiculite revealed little to no decreased persistence for any of the mutants, and in fact, survival in the surrounding soil was potentially increased for the *hyd2* mutant. Similarly, a double deletion Δ*Hyd1*Δ*Hyd3* in *C. rosea* resulted in increased root colonization compared to WT or single deletion of Δ*Hyd1 or* Δ*Hyd3* [160].

Our results indicated that hydrophobins affected radial growth and conidiation in *B. bassiana* but did not affect conidial germination. The partial restoration of growth rate and complete restoration of conidiation in rescued mutants indicated hydrophobins appear to be important for growth and conidiation. Prior studies similarly demonstrated the importance of *hyd* genes in growth and conidiation in other fungi. In *M. brunneum, hyd1, hyd2* or *hyd3* single mutants showed delayed and reduced conidiation compared to the WT parent. The study also showed that the deletion of *hyd1, hyd2* or *hyd3* genes did not affect conidial germination or appressoria formation [9]. Likewise, reduced conidiation has been reported for knockouts of either the *MPG1* or *MHP1* hydrophobins in *M. oryzae*, and for the Δ*HFB2* hydrophobin mutant in *T. reesei*. However, loss of specific hydrophobin genes can result in opposing phenotypes in different fungal species. For example, deletion of the *Hyd1* and *Hyd3* genes in *C. rosea* resulted in increased vegetative growth and increased conidiation [160]. Whereas deletion of *Hcf1* or *Hcf6* in another phytopathogenic fungus, *Cladosporium fulvum*, had no effects on either growth or conidiation under the conditions examined [170].

Aside from growth defects, inactivation of *B. bassiana hyd* genes resulted in increased ROS production in growing hyphae, indicating elevated oxidative stress, a finding that could potentially account for the reduced growth and conidiation phenotypes. The finding of increased ROS in the *hyd* mutants suggested that perturbation in cell wall structure and/or its integrity was due to oxidative stress. Although overall conidial yields were decreased in the mutants, no changes in the expression of the *FlbA*, *FlbB*, or *FlbD* conidiation genes were noted under a variety of growth conditions. In contrast, the *hyd* mutants failed to synthesize oosporein, a 1,4-dibenzoquinone implicated as an antimicrobial factor that suppresses competing microbes on the insect cadaver, allowing the fungus to grow and conidiate on the dead host [171, 172]. In this case,

loss of oosporein production correlated with decreased expression of the *pks9* required for its synthesis. Similar observations have been reported for *M. brunneum*, where deletion of *hyd1*, *hyd2* or *hyd3* reduced pigment production compared to the WT [9].

The *Mad1* and *Mad2* (*Metarhizium* adhesins 1 and 2) are specific adhesins expressed by *M. robertsii* that mediate conidial interactions with insect cuticle and plant surfaces, respectively [37]. Comparative analysis of the *Beauveria* genome revealed that *BBA_02419* and *BBA_02379* are orthologs of *Mad1* and *Mad2*, and have hence been named *bad1* and *bad2*, respectively [50]. On onion epidermis, there were no differences in germ tube formation between the *B. bassiana hyd* mutant strains in comparison to the WT; however, *bad2* expression was reduced in the hydrophobin mutant strains compared to the WT. The *bad2* gene was down regulated in deletion mutants compared to WT for all the conditions tested, whereas the expression of *bad1* was unaffected for deletion mutants. This correlation between the *bad2* gene and both hydrophobin genes suggested that the expression of *bad2* is somehow linked to the expression of both *hyd1* and *hyd2*. The low level of *bad2* expression could result in lower colonization of *hyd* mutants in comparison to WT. However, several possibilities can account for increased colonization of the Δ*hyd1/*Δ*hyd2* mutant on bean roots compared to the single mutants. Most notably, whereas the single mutant showed decreased expression of critical signal transduction pathway genes under a variety of conditions, this decrease was much lower (albeit still not up to WT levels) than Δ*hyd1/*Δ*hyd2* mutant. This data suggests that, in the double mutant, compensatory pathways may be involved due to cell wall stresses that may result from the loss of both hydrophobins.

Significant progress has been made in the characterization of signaling pathways and transcription factors that regulate stress responses and virulence in *B. bassiana* [173, 174]. In *B. bassiana*, *Bck1*, *Mkk1* and *Slt2* constitute the MAPK cell wall integrity pathway, which regulates

multiple developmental processes [66, 175]. The high-osmolarity glycerol (*Hog1*) pathway is also critical for stress response and other developmental programs and *B. bassiana* mutants in any of these components display affected growth, development, conidiation, virulence and/or stress susceptibilities [64]. In *B. bassiana*, *Hog1* regulates a novel mitochondrial oxidative stress response [176]. These pathways are also linked to the hydrophobins, with transcript levels of *hyd1* and *hyd2* down regulated in *B. cinerea Hog1* mutants [177]. Our data showed that the Δ*hyd1*/Δ*hyd2* mutant had elevated levels of *Hog1* expression in a variety of growth conditions, particularly those related to plant substrates, again linking cell wall structure/integrity to signaling pathways involved in oxidative stress. In contrast, expression of the *Bck1*, *Mkk1* and *Slt2* genes were down regulated in the Δ*hyd1* and Δ*hyd2* mutants, but less so in the Δ*hyd1*/Δ*hyd2* strain as compared to the WT. As this pathway regulates conidiation, the reduced expression of *Slt2* MAPK pathway could account for the decreased conidial yield observed for the hydrophobin mutants. In the plant necrotrophic fungus, *Botrytis cinerea*, *Slt2* similarly contributes to sporulation, vegetative growth, and plant tissue colonization [178]. Expression of enzymes including chitinases, and Ras GTPases were not altered in the *hyd* mutants as compared to the WT under all of the growth conditions tested indicating that these pathways are unaffected. Although, deletion of *hyd1* and *hyd2* genes appear to be involved in transcriptional regulation of signal transduction pathways (*Slt2* and *Hog1*), it cannot be disregarded that the relative stoichiometry of the MAPK pathways is not solely based on transcript or protein abundance but may be based on the overall phosphorylation of the MAPK cascade.

In conclusion, our data show that the *hyd1* and *hyd2* hydrophobins in *B. bassiana* contribute to plant root colonization and the rhizosphere competence of this fungus. These cell wall constituents may participate in mediating initial as well as consolidation of associations with the

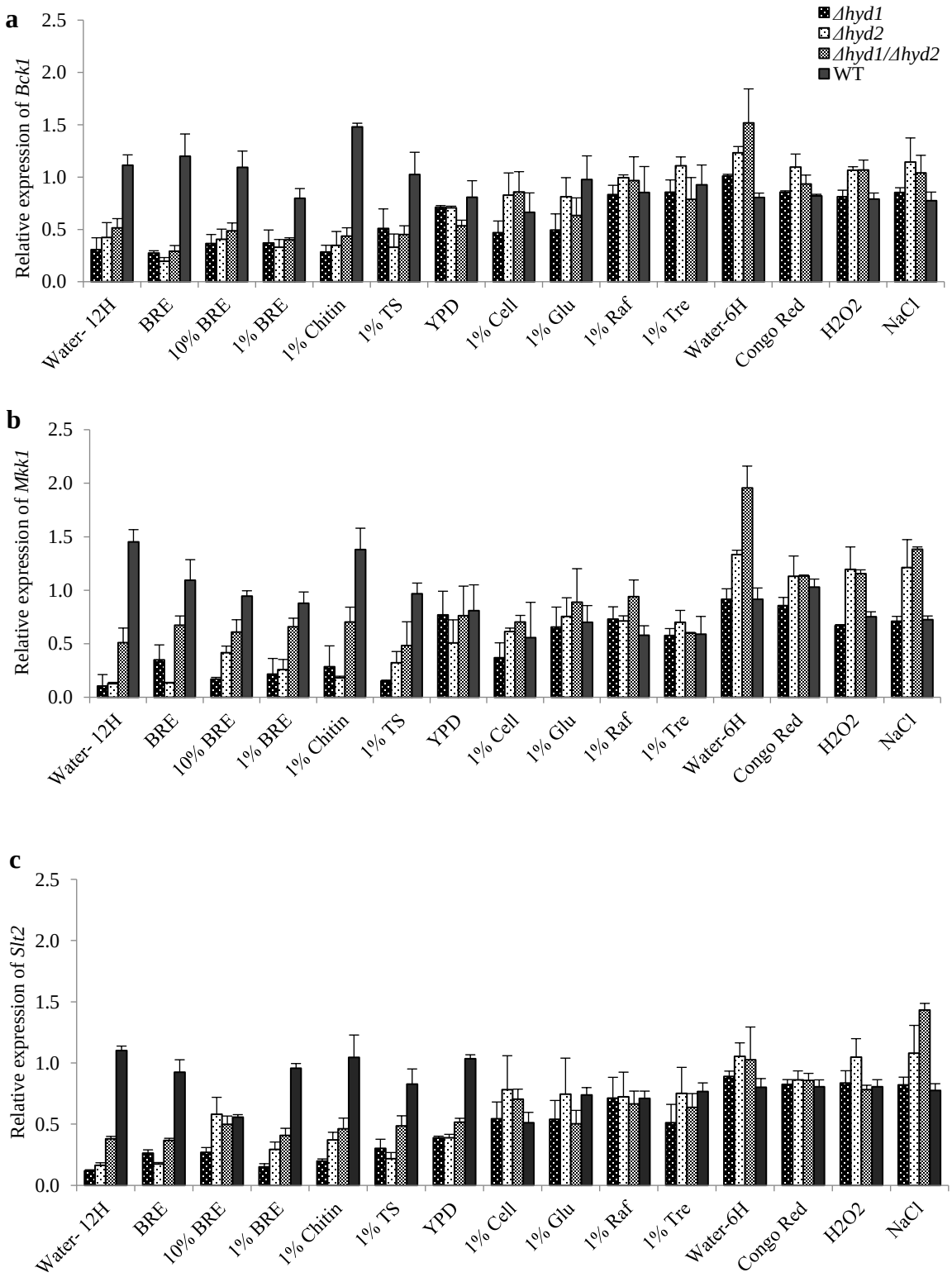
plant (root) surface. In contrast, *hyd1* had little impact on adhesion to insect cuticles but affected virulence, whereas *hyd2* appeared to have a significant role in mediating adhesion to the insect surface but contributed little to pathogenicity [10]. Hydrophobin effects were mediated, at least in part, via changes in the expression of specific MAPK signaling pathways, which correlated with downstream phenotypes including increased ROS production and oxidative stress seen in the mutants. These data indicate that rhizosphere interactions are specific and mediated by discrete pathways in *B. bassiana* and that some of these pathways, but not all, are shared with the ability of the fungus to parasitize insect hosts.

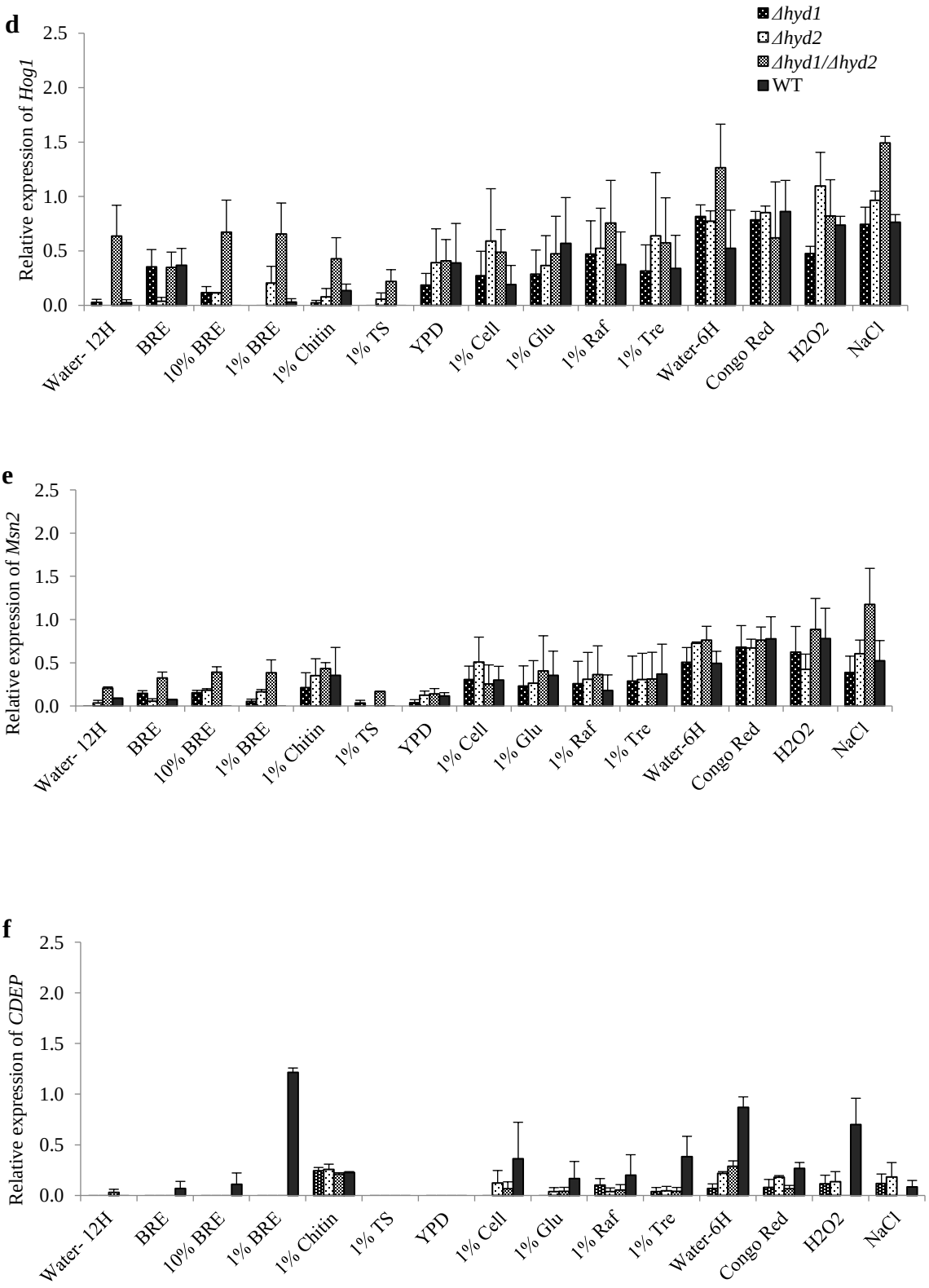
3.6 Supporting Information

Table S3.1 Primers used for semi-quantitative RT-PCR

Gene	Gene code	Annotation	Sequences (5′–3′) of paired primers
Conidiation			
FlbA	*BBA_02968*	Regulator of G-protein signalling (rgs)	CCACAACAACCACCACATTC /GTCCGCTGATGAGCTTGTCAC[1]
FlbB	*BBA_06988*	bZIP-type transcription factor	CACTGACACGCCGACAAGAG / TCGTAGCACCTCGTCCTCAAG[2]
FlbD	*BBA_07259*	MYB family conidiophore development protein	AATGCTATTGGTAGTGAAGG / CGACGATGTGGTGTAATG[2]
Cell wall integrity and insect virulence			
Chi1	*BBA_06317*	Chitinase	CGATTGGCGGTGCTACTG / AGGTTGGTCTGCGAGGTG[2]
Chit2	*BBA_10303*	Endochitinase	GTATGCCGCAGTGTTACC / AGAGCCGACTTCTGTTATTG[2]
ChsA2	*BBA_05353*	Class III chitinase	GGTAACGGCAACAAGATTG / TTTGGAGGAAAGCGAGTAG[3]
Ras Family (GTPase, Signal transduction)			
ras1	*BBA_04387*	Ras family small GTPase (Ras1)	GGCGGTGGCGTCGGTAAATC / TGGCGGGAGGTGATGGAGTA[4]
ras2	*BBA_04671*	Ras family small GTPase (Ras2)	CCTCGGTGACGGTGGTGTAG / GACTCCTTGACTCGCTGAATCTG[4]
ras3	*BBA_00036*	Ras family small GTPase (Ras3)	GCCTTTCTCCTCGTTTAC / CTCCATGTCGATCAAGTC[4]
Signal transduction network			
Hog1	*BBA_05209*	MAP kinase	TACTCTACGATTCGTCAAGT / TGCTGGAACAGAGCCGTCTT[5]
Bck1	*BBA_01318*	MAP kinase kinase kinase	ACTTGACCGCTTCGCCTATCG / GGCTGTCTTGAAGGTGGAATTGC[6]
Mkk1	*BBA_04254*	MAP kinase kinase	GCAATCATACACCATTACATC / ACTCCAGTAGACATCCATC[6]
Slt2	*BBA_03334*	MAP kinase	GAGGAGGTGCCCGAGATG / GCTCTGCTGCTGCTGTTG[6]
Adhesion or Hydrophobicity			
hyd1	*BBA_03015*	Hydrophobin	ATCTACTGCTGCAACGAGAA / TACTGGATAAGACTGCCAAT[5]
hyd2	*BBA_06599*	Hydrophobin	AGTGTCAAGACTGGCGACAT / ATCCGAGGACGGTGATGGGA[5]
bad1	*BBA_02419*	Adhesin protein *bad1*	GAAACAGCAACTTCCCCTGC / CTGGTTGCCCTGGTTTTTGG
bad2	*BBA_02379*	Adhesin protein *bad2*	ACCTCCCCCAGCTTCAGTC / TTACAGAAGAGCAGCGACGG
Subtilisin-like protease			
CDEP1	*BBA_00443*	Cuticle degrading protease	TGCACCGTCGGAGCTACCGA / AGTTGACGGTGCCTGAAGGA[5]
Oosporein			
pks9	*BBA_08179*	Polyketide synthase	TGCAAAGCGAGATCACCACT / GACACTTGCGGTGGGATGTA
Transcription factor (Involved in osmolarity glycerol pathway)			
Msn2	*BBA_00971*	Hog1-related transcription factor	GCCCCGCCACGCCCATCTAC / ACCGAGGTCTCAACCGAGTCAAAC[4]

[1] Fang et al. (2008); **[2]** Liu et al. (2015); **[3]** Liu et al. (2013); **[4]** Guan et al. (2015); **[5]** Zhang et al. (2009); **[6]** Chen et al. (2014).


a
Δhyd1
Δhyd2
Δhyd1/Δhyd2
WT
Relative expression of Bck1
2.5
2.0
1.5
1.0
0.5
0.0
Water- 12H
BRE
10% BRE
1% BRE
1% Chitin
1% TS
YPD
1% Cell
1% Glu
1% Raf
1% Tre
Water-6H
Congo Red
H2O2
NaCl
b
Relative expression of Mkk1
2.5
2.0
1.5
1.0
0.5
0.0
Water- 12H
BRE
10% BRE
1% BRE
1% Chitin
1% TS
YPD
1% Cell
1% Glu
1% Raf
1% Tre
Water-6H
Congo Red
H2O2
NaCl
c
Relative expression of Slt2
2.5
2.0
1.5
1.0
0.5
0.0
Water- 12H
BRE
10% BRE
1% BRE
1% Chitin
1% TS
YPD
1% Cell
1% Glu
1% Raf
1% Tre
Water-6H
Congo Red
H2O2
NaCl


d
Δhyd1
Δhyd2
Δhyd1/Δhyd2
WT
Relative expression of Hog1
2.5
2.0
1.5
1.0
0.5
0.0
Water- 12H
BRE
10% BRE
1% BRE
1% Chitin
1% TS
YPD
1% Cell
1% Glu
1% Raf
1% Tre
Water-6H
Congo Red
H2O2
NaCl
e
Relative expression of Msn2
2.5
2.0
1.5
1.0
0.5
0.0
Water- 12H
BRE
10% BRE
1% BRE
1% Chitin
1% TS
YPD
1% Cell
1% Glu
1% Raf
1% Tre
Water-6H
Congo Red
H2O2
NaCl
f
Relative expression of CDEP
2.5
2.0
1.5
1.0
0.5
0.0
Water- 12H
BRE
10% BRE
1% BRE
1% Chitin
1% TS
YPD
1% Cell
1% Glu
1% Raf
1% Tre
Water-6H
Congo Red
H2O2
NaCl

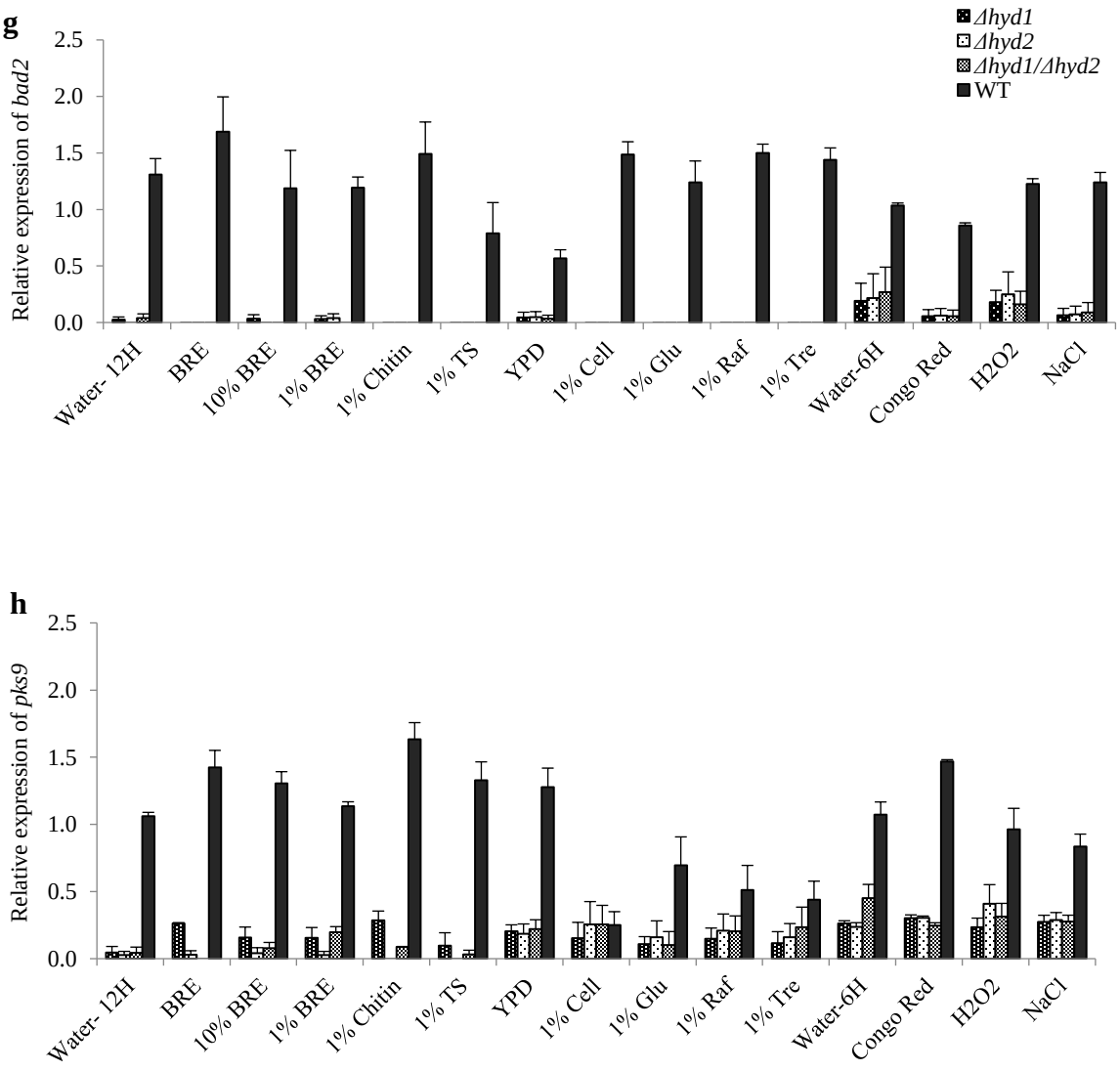


Figure S3.1. <u>Relative expression of selected genes under different conditions for WT and *hyd* mutant strains</u>. a. *Bck1*, **b.** *Mkk1*, **c.** *Slt2*, **d.** *Hog1*, **e.** *Msn2*, **f.** *CDEP*, **g.** *bad2*, **h.** *pks9*. The error bars represent standard error. BRE - bean root exudate, TS- tomato stem, YPD – yeast extract dextrose broth, Cell – cellulose, Glu – glucose, Tre - trehalose, Raf- raffinose, H2O2 – hydrogen peroxide, NaCl – sodium chloride.

Chapter 4 - *Metarhizium robertsii* ammonium permeases (MepC and Mep2) contribute to rhizospheric colonization and modulates the transfer of insect derived nitrogen to plants

Authors: Soumya Moonjely, Xing Zhang, Weiguo Fang and Michael J. Bidochka

4.1 Abstract

Here we assessed the involvement of six *M. robertsii* genes in endophytic, rhizoplane and rhizospheric interactions with barley roots. Two ammonium permeases (*MepC* and *Mep2)* and a *Urease*, were selected since homologous genes in arbuscular mycorrhizal fungi were reported to play a pivotal role in nitrogen mobilization during plant root colonization. Three other genes were selected on the basis of RNA-seq data that showed high expression levels on bean roots, and these encoded a hydrophobin (Hyd3), a subtilisin-like serine protease (Pr1A) and a hypothetical protein. Two previously characterized genes, *Mad2* and *Mrt*, identified as a plant specific adhesin and a raffinose transporter, respectively, were also included in the assay as controls. The root colonization assays revealed that the deletion of *MepC*, *Mep2, Urease*, *Hyd3, Pr1A, hypothetical protein*, *Mrt* or *Mad2* had no impact on endophytic colonization at 10 or 20 days. However, the deletion of *MepC* and *Mrt* resulted in increased rhizoplane colonization at 10 days whereas Δ*Mep2* showed increased rhizoplane colonization at 20 days. The enhanced root colonization of Δ*Mep2* and Δ*MepC* is possibly due to the effect of ammonium permease regulation in response to nitrogen availability. No changes in rhizoplane colonization relative to WT at 10 or 20 days were found for Δ*Urease*, Δ*Hyd3*, Δ*Pr1A*, Δ*hypothetical protein* or Δ*Mad2*. The nitrogen transporter mutants showed higher ^{15}N incorporation compared to WT in MMN treated barley plants after 10 days of growth in the presence of microcosms containing ^{15}N-injected wax moth larvae. Colony growth

characteristics on different nitrogen sources showed growth impairment with the Δ*MepC* on media without nitrogen supplementation or low nitrogen conditions (1mM NH4 and 1mM proline). The targeted deletion of *Hyd3* and *Pr1A* decreased virulence against *Tenebrio molitor* larvae but was unchanged compared to WT against *Galleria mellonella* larvae.

4.2 Introduction

The endophytic, insect pathogenic fungi (EIPF) *M. robertsii* (Clavicipitaceae) exhibits a varied lifestyle as an entomopathogen, endophyte or as a saprophyte [161]. *Metarhizium* spp. have been widely used as biocontrol agents against insect pests in agricultural fields. The molecular and biochemical factors regarding insect pathogenicity are relatively well studied [3, 162]. Several *Metarhizium* spp. colonize the plant root and form a beneficial symbiotic relationship [1]. However, the genetic factors underpinning plant root colonization or rhizospheric competence in *M. robertsii* is largely unexplored.

M. robertsii forms a close symbiotic association with certain plants and is capable of transferring insect-derived nitrogen [6, 89] to the plant in exchange for carbon [179]. The importance of a sugar transporter (*Mrt)* [98] and invertase (*MrINV*) [99] during *Metarhizium*-plant interaction has been demonstrated. Nevertheless, genes involved in nitrogen transfer in *M. robertsii* during plant symbiosis are unknown. Nitrogen is a major soil-limiting nutrient and is integral for photosynthesis. Most plants rely on nitrogen fixing microorganisms or microbial decomposers to acquire nitrogen from the soil. The involvement of nitrogen transporters, ammonium permease and urease, are reported in arbuscular mycorrhizal (AM) fungi during plant colonization [106, 180–183]. AM fungi acquire nitrogen from the soil in the form of ammonium,

nitrate or amino acids, which are then transported to host plants through ammonium /methylammonium permease (AMT/Mep) transporters. Here inorganic nitrogen or ammonia is assimilated by AM fungi and subsequently converted to arginine in the extraradical mycelium by nitrate reductase or glutamine synthase/glutamate synthase. Arginine is then translocated to the intraradical mycelium where it is converted to urea and ornithine. Urea is then converted to NH_4^+ by the action of urease, which is subsequently mobilized to the plant via ammonium transporters/permeases [106]. *Metarhizium* share similarities with mycorrhizal fungi in terms of forming symbiotic association and facilitating nutrient exchange with plants, hence it is hypothesized that similar nitrogen transfer mechanisms may be operating. A genome survey of *M. robertsii* revealed the presence of two ammonium permeases (*MepC* and *Mep2*) and a urease gene.

Metarhizium expresses different subsets of genes as a means of physiological adaptation under various conditions. Expressed sequenced tag and cDNA microarray analysis have demonstrated large scale differences in expression patterns during growth in plant root exudate, insect cuticle or hemolymph. For example, 18 out of 43 genes were found differentially expressed in insect cuticle and in root exudate; one major exception was a subtilisin-like protease (*Pr1A*) which was highly expressed under both conditions [71, 184]. Data from comparative RNA-Seq transcriptome analysis provided insights into the evolution and pathogenicity of generalist and specialist *Metarhizium* species [11, 87]. Although the symbiotic ability and the role as a plant growth promoter has been demonstrated, little is known about the genes involved in this symbiosis.

In order to get a better understanding of the genes involved during *Metarhizium*-plant symbiosis, RNA-Seq was performed from *Metarhizium* transcripts during bean root colonization

and three genes that showed high expression levels were selected for further analysis. Two ammonium permeases (*MepC* and *Mep2*) and a *urease* gene were also selected. Over all, we investigated the involvement of 6 different genes *MepC* (*MAA_04182), Mep2* (*MAA_05002*), *Urease* (*MAA_07458), Pr1A* (*MAA_05675*), *Hypothetical protein* (*MAA_08959*), *Hydrophobin 3* (*MAA_10298)* in *M. robertsii* during barley root colonization and insect pathogenesis, by using targeted gene deletions. *M. robertsii* adhesin2 deletion (Δ*Mad2*) and a raffinose transporter deletion (Δ*Mrt)* were used as comparisons for the plant root colonization assays. In addition, we also quantified the ability to transfer insect-derived nitrogen to barley plants by Δ*MepC*, Δ*Mep2* and Δ*Urease* mutants.

4.3 Materials and Methods

4.3.1 Fungal strains and culture conditions

Metarhizium robertsii (ARSEF 2575) wild-type (WT) strain was grown and maintained on Potato Dextrose Agar (PDA) (BioShop Canada Inc., Burlington, ON) at 27^{o}C. *M. robertsii* ammonium permease C deletion (Δ*MepC*,) ammonium permease 2 deletion (Δ*Mep2*), Δ*Urease*, subtilisin-like protease deletion (Δ*Pr1A*), Δ*Hypothetical protein (*Δ*Hypo. protein)* and hydrophobin deletion (Δ*Hyd3*) were used in this study. *M. robertsii* adhesin 2 deletion (Δ*Mad2*) [37] and a raffinose transporter deletion (Δ*Mrt)* [98] were used as comparisons for the plant root colonization assays and were kindly provided by Dr. R.J. St. Leger (University of Maryland). The fungal cultures were routinely grown and maintained on PDA as needed. Conidia were obtained from 12-14 day old PDA plates and conidia were recovered with 0.01% Triton X-100. Conidial yields were then quantified using a hemocytometer and the concentration adjusted to $1x10^{7}$ conidia/mL.

4.3.2 Targeted gene deletions

Gene deletions based on homologous recombination was conducted according to our previously developed high-throughput methodology [185]. The plasmids for gene deletion were constructed using Gateway Bp Clonase II Enzyme Mix (Invitrogen, Carlsbad, CA) or restriction enzyme digestion and ligation. The primers for deletion plasmid construction and other primers used in this study are presented in Supporting information (Table S4.1). All PCR products were cloned using high-fidelity Taq DNA polymerase (KOD) and confirmed by DNA sequencing. PCR was performed (Table S4.1) to verify the targeted gene deletions (Figure S4.1).

4.3.3 Growth rate, conidial germination and stress sensitivity assay

The radial growth of *M. robertsii* WT and gene deletion strains was examined by inoculating the center of the PDA plate with 10 µL of the conidial suspension ($1x10^7$ conidia/mL), incubated at 27^{o}C and colony diameter was measured on day 3, 7 and 14. For the stress sensitivity assay, 10 µL of the conidial suspension ($1x10^7$ conidia/mL) was inoculated on PDA plates supplemented with either 0.01% SDS or 100 µg/mL Congo red. The plates were incubated at 27^{o}C in the dark and the colony diameters were recorded after 7 days [10].

The hydrophobicity of Δ*Hyd3* was assessed using a wettability test [9]. The test was performed by placing 10 µL of a water droplet on the surface of a 14 day old culture and the contact angle of the water droplet was observed after 10 minutes, 30 minutes and 1 hour.

4.3.4 RNA sequencing of *Metarhizium* associated plant root

Briefly, *M. robertsii* were allowed to associate with bean roots for 7 days thereafter the root was washed, macerated and RNA extracted. cDNA was prepared and sequenced with the

SOLiD™ 4 (Life Technologies, Carlsbad, CA) system. *Metarhizium* transcripts were identified and differentiated from plant transcripts by transcript comparison to the *Metarhizium* genome as the template.

4.3.5 Root colonization assays

Root colonization assays were performed using barley (*Hordeum vulgare*, Sprout Master, Elmvale, ON) as the host plant. The endophytic, rhizoplane and rhizospheric association of WT and gene deletion strains were analyzed. Seeds were surface sterilized with three washes in 4% sodium hypochlorite (NaOCl) for 5 minutes each. The seeds were rinsed with sterile distilled water after each NaOCl wash. The seeds were kept overnight at 4°C for synchronization of growth before planting. The seeds were then allowed to germinate in water agar for 3-4 days at 25°C. The germinated seedlings were then planted in sterile vermiculite (Ther-O-Rock East Inc., New Eagle, PA). Fungal inoculations were performed using the drench method [186] where 5 mL of the conidial suspension was poured onto the vermiculite surface of each pot. The plants were kept in a greenhouse at 25°C with a photoperiod of 16:8 h light: dark cycle with relative humidity maintained between 60-80%. The plants were watered daily with sterile distilled water. Five biological replicates were prepared for each treatment. To quantify fungal association, barley roots were harvested from 10 and 20 day old plants. The amount of fungal association on barley roots was analyzed as described previously [187]. To examine the endophytic association, the harvested roots were first washed in water to remove the attached vermiculite. The washed roots were then immersed in 2%NaOCl for 10 seconds and finally rinsed with sterile distilled water to remove traces of NaOCl. The roots were then cut into ~2-5mm pieces, weighed and homogenized (Biospec products Inc., Bartlesville, OK) in sterile distilled water for 2 mins. The homogenized root samples

were then plated on modified CTC agar (PDA supplemented with 0.5 g/L chloramphenicol, 0.004 g/L thiabendazole and 0.5 g/L cycloheximide) [166] and CFU values were calculated as CFU/g of root weight. To check the rhizoplane colonization, the harvested roots were treated as described above except the 2% NaOCl treatment was omitted. Water inoculated barley plants were used as controls. Rhizospheric populations of WT and gene deletion strains were also monitored. Here, the vermiculite attached to the barley roots was collected during the harvest, weighed and suspended in 0.01% Triton X-100. The serial dilutions of the suspension were plated on CTC media and the CFU were calculated for per gram of vermiculite.

4.3.6 Insect bioassays

The virulence of the WT *M. robertsii* and gene deletion strains were assayed against larvae of *Tenebrio molitor* and *Galleria mellonella*. An aliquot of 10 µL of the conidial suspension ($1x10^7$ conidia/mL) was applied to the cuticle of the larvae. Each larva was placed separately in 60mm x 15mm diameter Petri dishes. Humidity was maintained in each Petri dish with a moistened filter paper. The treated larvae were housed at 25^{o}C and daily mortality rate was recorded. Each replicate contained 20 larvae and the experiment was duplicated. Controls were 0.01% Triton X-00 treated larvae. The LT50 values were calculated using Probit analysis.

4.3.7 ^{15}N-labelled nitrogen transfer assays

The ability of Δ*MepC*, Δ*Mep2* and Δ*Urease* to translocate nitrogen from ^{15}N -labeled wax moth larvae to the leaves of barley was quantified by the microcosm method as previously described [6, 89]. A 10 µL solution of ^{15}N-labelled ammonium sulphate (5%) was injected trough the rear proleg of wax-moth larvae. After 48 hours the larvae were infected with 14 day old fungal

conidia. The infected larvae were then placed into microcosms containing autoclaved vermiculite as previously described [89]. The microcosm was then placed in the pots and covered with autoclaved vermiculite and the 3 day old germinated barley seedling was planted on each pot. The ^{15}N transfer was assessed by the nitrogen transporter mutant strains using two treatments. In the first experimental set plants were watered daily with sterile distilled water and once a week with 25 mL of 50% MMN solution (0.05 g $CaCl_2$, 0.025 g NaCl, 0.05 gKH_2PO_4, 0.5 g $(NH4)_2PO_4$, 0.15 g $MgSO_4 \cdot 7H_2O$, 1 mg $FeCl_3 \cdot 6H_2O$,5g glucose monohydrate, 10 mL trace element solution [3.728 g KCl, 1.546 g H_3BO_3, 0.845 g $MnSO_4 \cdot H2O$, 0.05 g $ZnSO_4 \cdot 7H_2O$, 0.0125 g $CuSO_4$, 0.05 g $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ per 1 liter] per 1 liter). In the second treatment, plants were watered daily with sterile distilled water but 50% MMN solution was omitted. The amount of ^{15}N transfer to plant tissues was determined by harvesting the above ground plant parts after 10 and 20 days. The harvested plant material was dried at 60°C for 24 hours and were then crushed into a fine powder using a mortar and pestle. The ground plant material was encapsulated in 4-mm by 4-mm tin cups and analyzed for ^{15}Ncontent by using an NOI-5 emission spectrophotometer.

4.3.8 Nitrogen source assays

The ability of Δ*MepC,* Δ*Mep2,* Δ*Urease* and WT to grow on different nitrogen sources was assessed on basal salt agar (BS; 0.1% KH_2PO_4, 0.025% Na_2SO_4, 0.05% KCl, 0.0125% $MgSO_4 \cdot 7H_2O$, 0.00625% $CaCl_2$, and 1% glucose) [98] supplemented with different nitrogen sources (1 or 30mM) $(NH_4)_2$ SO_4, 30 mM arginine, 30 mM glutamine, 30 mM glutamate, 1 or 30 mM proline or 30mM urea). A $1x10^7$ conidial/mL suspension (5 μL) was spot inoculated onto BS agar supplemented with different nitrogen sources and growth rates were compared with the WT after 7 days.

Ammonium production, based on pH of the mutant strains and WT, was also examined. Here, 1 mL of the conidial suspension was added on to potato dextrose broth and allowed to grow for 4 days at 100 rpm at 27^{o}C. Mycelia was then filtered, washed with sterile distilled water and transferred to minimal media (0.02% KH_2PO_4, 0.01% $MgSO_4$, 0.2ppm $FeSO_4$, 1 ppm $ZnSO_4$, 0.02 ppm $CuSO_4$, 0.02 ppm $MnMoO_4$, 0.02 ppm $MnCl_2$) supplemented with different amino acids (10 mM glutamate, glutamine or arginine). The fungal mycelia (2.5g) was added to 100 mL of minimal media broth and incubated at 100 rpm at 27^{o}C. The samples were collected at 0, 6, 24 and 48 hours and the pH recorded.

4.3.9 Phylogenetic analysis

Sequence alignments were conducted using MUSCLE v3.7 with default parameters [188], and these were then manually refined and end-trimmed to eliminate poor alignments and divergent regions. Unambiguously aligned positions were used for constructing phylogenetic trees with Maximum Likelihood (ML), Bayesian Inference (BI) or distance-based Neighbor-Joining (NJ). A ML tree was constructed using MEGA6.0 (gap treatment: use all sites; 100 bootstrap replications) [189]. The optimal model of phylogenetic relationship was determined using the Find Best Protein Model provided by MEGA 6.0 [189]. A Bayesian inference tree was constructed with MrBayes v3.2.5 as described [190]. The best model was determined as above using MEGA 6.0. For each BI analysis, we used four Metropolis-coupled chains and ran them for 5,000,000 generations, sampling every 1000 generations ('mcmc ngen = 5000000 sample frequency =1000'). The analysis finished with an average standard deviation of split frequencies of 0.01 or less. The first 25% of trees were discarded as "burn-in". A NJ tree was constructed with default parameters (gap

treatment: pairwise deletion; 1000 bootstrap replications) using NJ in MEGA6.0 [189]. Bootstrap support values were obtained by generating 1,000 pseudo-replicates.

4.4 Results

4.4.1 RNA sequencing and transcriptome analysis

RNA sequencing (RNA-seq) of *Metarhizium* transcripts during bean root association revealed the expression of over 4000 *Metarhizium* genes. In transcriptome data, 217 genes showed relatively higher expression (10-234 readings) and 25% of these upregulated genes were putative uncharacterized/ hypothetical protein. Of these, the top five *M. robertsii* genes that showed highest expression levels (~90-234 readings) were hydrophobin (*MAA_10298*), tubulin beta chain (*MAA_02081*), subtilisin-like serine protease *Pr1A* (*MAA_05675*) and two other genes that encode putative uncharacterized proteins (*MAA_08959* and *MAA_00771*). The protein sequence of *MAA_08959* (*hypothetical protein*) was surveyed in GenBank, and similar sequences were reported in other *Metarhizium* species, however these were also categorized as uncharacterized/hypothetical protein. A homolog of *MAA_08959* was reported in *M. brunneum* as filamin/ABP280 repeat like protein but was not functionally characterized. Table 4.1 shows the top ten *Metarhizium robertsii* genes that showed increased expression in bean root (RNA seq of <1% of total transcripts).

Table 4.1. The top 10 *Metarhizium robertsii* genes that showed increased expression in *Glycine max* root (RNA seq of <1% of total transcripts).

#	Identification	Id Gene	Number of readings
1	*Putative uncharacterized protein*	*MAA_08959*	234
2	*Hydrophobin*	*MAA_10298*	231
3	*Tubulin beta chain*	*MAA_02081*	155
4	*Putative uncharacterized protein*	*MAA_07571*	131
5	*Subtilisin-like serine protease PR1A*	*MAA_05675*	96
6	*Putative uncharacterized protein*	*MAA_09351*	88
7	*Putative uncharacterized protein*	*MAA_08500*	86
8	*Putative uncharacterized protein*	*MAA_09173*	86
9	*Mmc protein*	*MAA_02991*	77
10	*Hydrophobin-like protein ssgA*	*MAA_09731*	65

4.4.2 Phylogenetic analysis

BLAST search using the NCBI protein database of MepC showed amino acid sequence similarities to the ammonium transporters of phytopathogens and other insect pathogens/plant root colonizers, with more than 80% similarity to other Hypocreales such as *Ustilaginoidea virens*, *Beauveria bassiana* and *Trichoderma harzianum* (Figure 4.1). These fungi represent diverse lifestyles as phytopathogens, endophytic insect pathogens and endophytic mycoparasites, respectively. Phylogenetic analysis showed that Mep2 amino acid sequences clustered with ammonium permeases from phytopathogenic and endophytic fungi. Mep2 showed a close phylogenetic relationship with ecto/endo mycorrhizal ammonium transporters, while MepC formed a distant clade with mycorrhizal ammonium transporters.

The BLAST analysis of urease showed more than 80% amino acid sequence similarities with *Purpureocillium lilacinum* (entomopathogenic, rhizospheric), *Tolypocladium capitatum* (insect parasitic, mycoparasitic), *Hirsutella minnesotensis* (insect and nematode pathogenic) and other endophytic/phytopathogenic fungi (*Trichoderma* sp., *Colletrotrichum* sp., *Verticillium* sp. and *Fusarium* sp.). The nucleic acid sequence analysis of *MAA_10298* revealed sequence identity with a functionally characterized Class I hydrophobin gene from *M. brunneum*, *HYD3*, and hence named as *Hyd3* in this study.

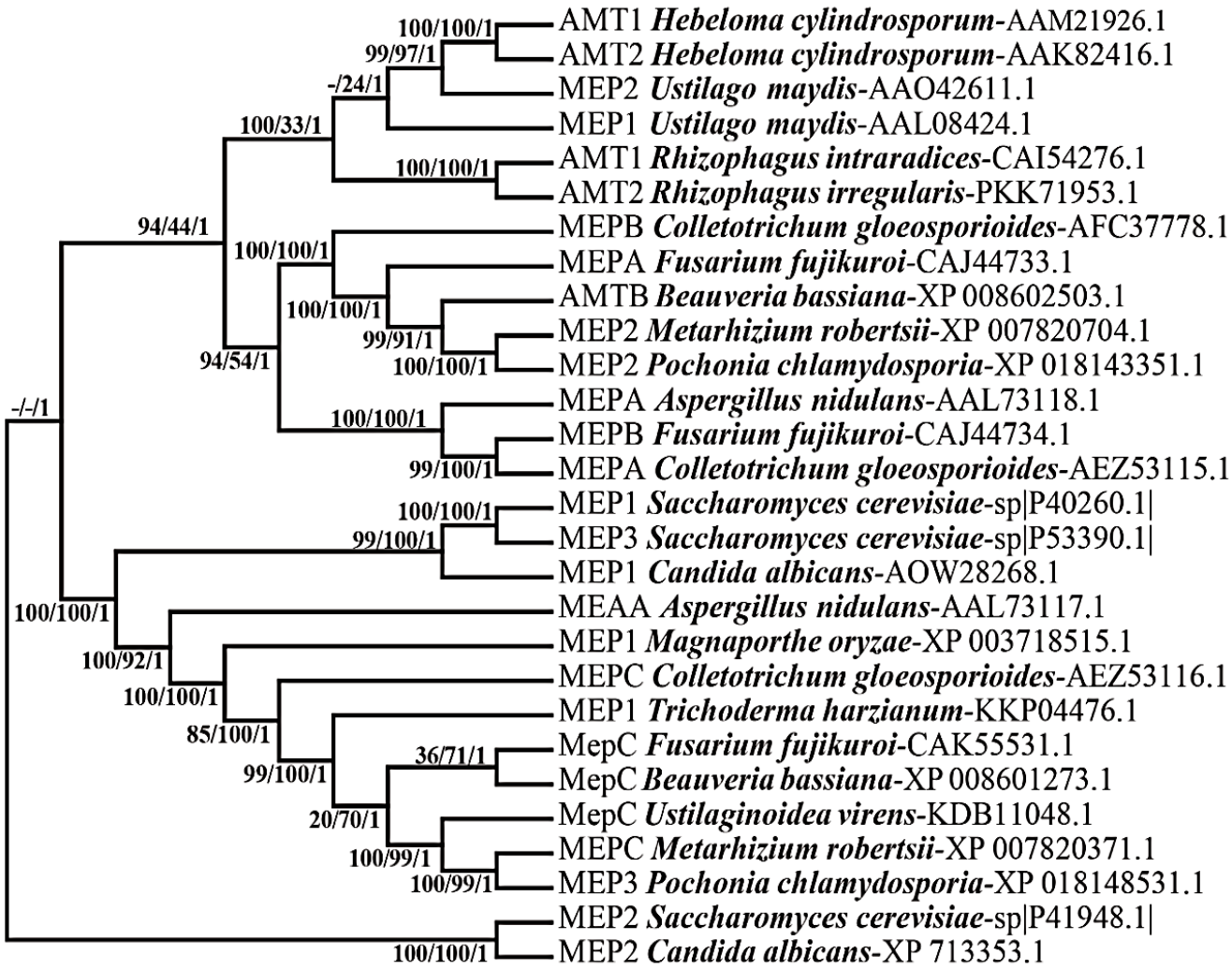


Figure 4.1. **Phylogenetic relationship between the amino acid sequences of ammonium permeases of *M. robertsii* and other fungal ammonium transporters.** The phylogenetic tree was created using Maximum Likelihood (ML), Bayesian Inference (BI) and Neighbor joining (NJ). The Mep2 of *Saccharomyces cerevisiae* sequence was used to root the tree. The sequences of the other fungal transporters were obtained from GenBank database which is as follows: *Aspergillus nidulans* (AnMEAA: AAL73117,AnMEPA: AAL73118), *Beauveria bassiana* (MEP3: PMB65720.1), *Candida albicans* (MEP1: AOW28268.1, MEP2: XP 713353.1), *Colletotrichum gloeosporioides* (MEPA: AEZ53115.1, MEPB: AFC37778.1, MEPC:AEZ53116.1), *Fusarium fujikuroi* (FfMEPA: CAJ44733,FfMEPB: CAJ44734, FbMEPC: CAK55531), *Glomus intraradices* (GintAMT1: CAI54276; GintAMT2:CAX32490), *Hebeloma cylindrosporum* (HcAMT1: AAM21926, HcAMT2: AAK82416, HcAMT3: AAK82417), *Metarhizium robertsii* (MepC: XP_007820371.1, Mep2:XP007820704.1), *Rhizophagus irregularis* (AMT2: PKK71953.1), *Rhizophagus intraradices* (AMT1: CAI54276.1), *Saccharomyces cerevisiae* (ScMEP1: P40260, ScMEP2: P41948, ScMEP3: P53390), *Trichoderma harzianum* (MEP1 KKP04476.1), *Ustilago maydis* (UmMEP1: AAL08424, UmMEP2: AAO42611), *Ustilago virens* (MepC; KDB11048.1).

4.4.3 Effect of gene disruption on phenotypic characteristics

The growth rate of WT *M. robertsii* and the mutant strains were similar on PDA at 27^{o}C (Figure 4.2a). However, subtle differences in the colony morphology were noted in gene deletion strains relative to WT. The Δ*Hypo. protein* showed a thick white cottony mycelium (Figure 4.2c) compared to WT on PDA. The effect of various stress conditions on the growth of mutant strains was assayed on agar plates containing either Congo red or SDS. No differences in the growth rate were observed in any of the stress conditions (Figure 4.2c). Disruption of *Hyd3* affected conidiation on PDA and conidial yield was decreased by ~7 fold compared with WT (ANOVA, $p<0.001$) (Figure 4.2b). No differences in conidiation were noted for other gene deletion strains relative to WT (Figure 4.2b).

The hydrophobicity test showed that Δ*Hyd3* was a wettable phenotype observed as a decrease in the contact angle of the water droplet on the colony surface (Figure 4.2d). The WT retained the water droplet after 1 hour while Δ*Hyd3* exhibited a less hydrophobic colony surface.

4.4.4 Effect of gene disruption on insect pathogenesis

Pathogenicity assays against *Galleria mellonella* larvae revealed no reduction in the mortality by mutant strains (Figure 4.3a). Similar results were obtained for *Tenebrio molitor* larvae bioassays except that Δ*Pr1A* LT50 5.90±0.28 and Δ*Hyd3* LT50 6.02±0.56 showed reductions (ANOVA, $p = 0.04$) in LT50 relative to the WT (LT50 4.53±0.1) (Figure 4.3b).

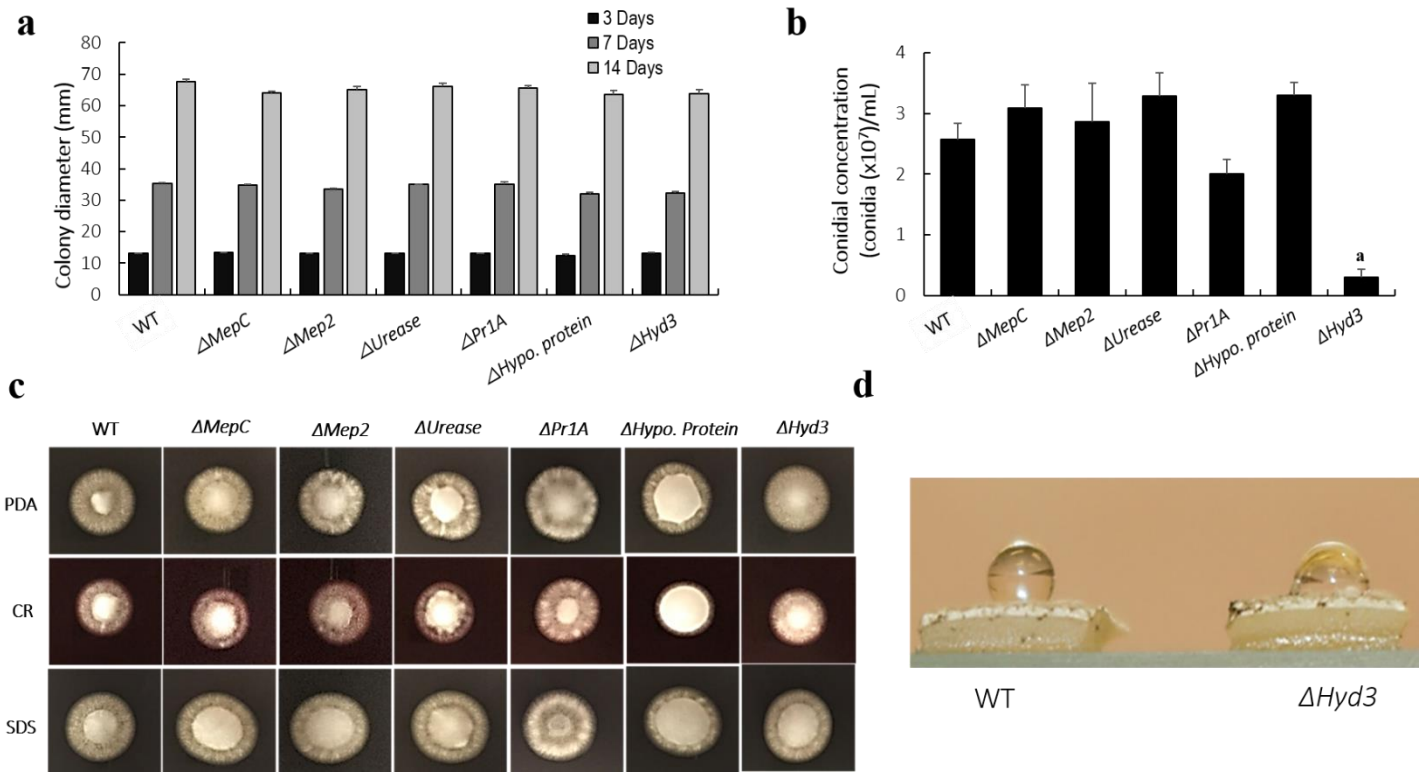


Figure 4.2 a. Growth rate of *M. robertsii* and mutant strains on potato dextrose agar medium. 10µL of the $1x10^6$ conidia/mL was spot inoculated on solid agar medium and incubated at 27°C. The colony diameter was measured on 3, 7 and 14 days. The error bars represent the standard error based on 5 biological replicates. **b. Quantification of conidial production of *M. robertsii* and mutant strains.** Different letters indicate statistically significant differences from WT, 'a' indicates $p<0.001$. **c. Colony morphology** of WT and mutants in PDA and response stress conditions, 0.01% SDS, 100 µg/mL Congo red (CR). **d. Water droplet hydrophobicity test of WT and Δ*Hyd3*.** Image shown was taken after 10 mins. The difference in the contact angle of the water droplet is wide for Δ*Hyd3* compared to WT.

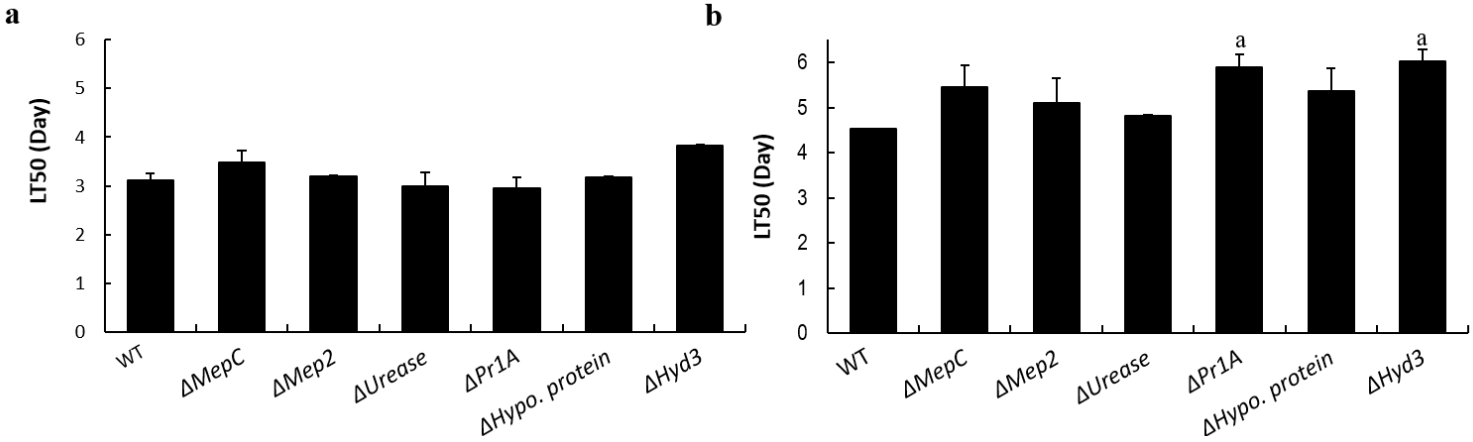


Figure 4. 3. Insect bioassays. The calculated LT50 values for *M. robertsii* WT and mutants on topical application to **a.** *Galleria mellonella*, **b.** *Tenebrio molitor*. The error bar represents the standard deviation of two replicates and each replicate contained 20 larvae. Different letters indicate statistically significant differences from WT, 'a' indicates $p<0.05$.

4.4.5 Effect of gene disruption on rhizospheric and endophytic competency

The ammonium permease deletions, Δ*MepC* and Δ*Mep2* showed increased rhizoplane colonization after 10 or 20 days respectively (Figure 4.4a). An increase in CFU was observed for Δ*MepC* ($24.5x10^4$ CFU/g of root) at 10 days post treatment as compared to WT ($7.1x10^4$ CFU/g of root) (2-way ANOVA, Tukeys's multiple comparison test: WT v/s Δ*MepC* ($p<0.001$)). However, Δ*MepC* showed no differences in rhizoplane colonization levels relative to WT after 20 days. In contrast, *ΔMep2* showed similar levels of root colonization as the WT after 10 days; however, higher CFU levels ($35.5 x10^4$ CFU/g of root) was recovered from after 20 days (WT v/s *ΔMep2* ($p<0.0001$)). The sugar transporter deletion Δ*Mrt* showed a similar rhizoplane colonization pattern as Δ*MepC*. Rhizoplane colonization for Δ*Mrt* ($27.7x10^4$ CFU/g of root) after 10 days post treatment was ca. 4 times greater than WT. Nevertheless, the recovery of Δ*Mrt* ($4.3x10^4$ CFU/g of root) from plant roots after 20 days post inoculation was similar to the WT ($3.6x10^4$ CFU/g of

root). The disruption of the urease gene in *M. robertsii* had little impact on root colonization. Similarly, the disruption of *Hyd3*, *Pr1A* and *Hypo. protein* did not affect the rhizospheric or rhizoplane colonization abilities compared with the WT. When the rhizospheric persistence of these strains in vermiculite was analyzed, there were no differences in the CFU recovered from vermiculite compared to WT except for *ΔMepC* at day 10 (Figure 4.4b). No differences in endophytic colonization was observed for mutants compared with the WT (Data not shown).

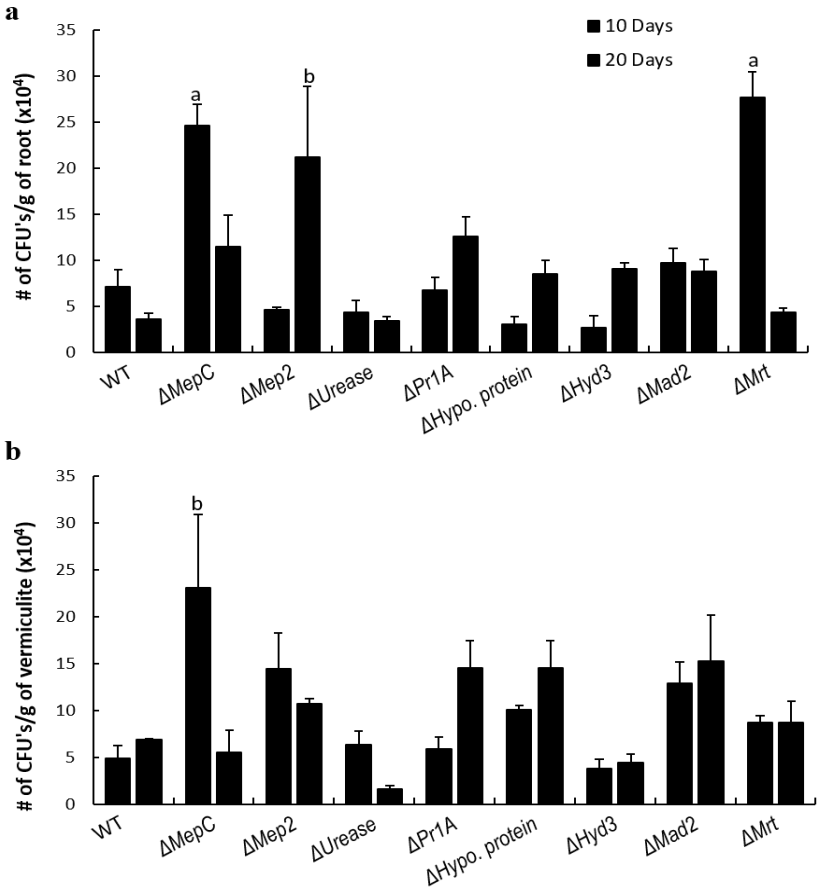


Figure 4.4. Rhizoplane and rhizosphere colonization of *M. robertsii* WT and mutant strains. a. Rhizoplane colonization. 3- 4 days old germinated barley seedlings were planted on sterile vermiculite inoculated with 5 mL of 10^7 conidia/mL. Barley roots were harvested on 10 and 20 days post inoculation and washed in water. The roots were then homogenized and plated on selective media plates. The CFUs were counted after 7 days and CFUs/g of root weight was calculated. **b. Rhizosphere colonization.** ~1±0.25 g of vermiculite surrounding root was collected on the day of 10 and 20 day harvest. Vermiculite was suspended in 0.01% Triton X-100 and 0.1 mL was plated on selective media after serial dilution. The CFU's were counted after 7 days and CFUs/g of soil weight was calculated. The error bars represent the standard error for 5 biological replicates and different letters indicate statistically significant differences relative to WT. 'a', $p<0.008$; 'b', $p<0.02$.

4.4.6 Insect derived ^{15}N transfer to barley by *Metarhizium* nitrogen transporter mutants

Metarhizium strains including WT and the nitrogen transporter mutants were able to transfer significant amounts of insect derived-^{15}N to barley after 10 and 20 days of growth (Figure 4.5). *M. robertsii* WT showed difference in the insect derived-^{15}N incorporation between MMN treated and untreated plants after 10 days of growth in microcosms. Decreased ^{15}N incorporation was noted in MMN treated plants (68.84%) versus untreated plants (84.33%) when grown in microcosms containing *M. robertsii* WT infected, ^{15}N injected wax moth larvae. However, no difference was observed in insect derived-^{15}N incorporation between MMN treated and untreated after 20 days of growth in the presence of WT infected, ^{15}N-injected wax moth larvae.

All three of the nitrogen transporter mutants showed higher ^{15}N incorporation compared to WT in MMN treated barley plants after 10 days of growth in the presence of microcosms containing ^{15}N-injected wax moth larvae. After 10 days of growth, Δ*MepC* (81.25%) showed higher insect derived-^{15}N transfer to barley leaves relative to the WT (68.84%) in MMN treated plants. In contrast, plants that were not treated with MMN, Δ*MepC* (79.75%) showed less ^{15}N transfer compared to WT (84.33%) (2-way ANOVA, Tukeys's multiple comparison test: WT v/s Δ*MepC* ($p<0.03$)). However, no difference in insect derived-^{15}N incorporation by Δ*MepC* was observed relative to WT in both MMN treated and untreated plants after 20 days of growth.

In the MMN treated barley plants, Δ*Mep2* showed greater ^{15}N transfer to barley for both 10 (78.10%) and 20 (82.95%) days compared to WT (68.84% and 77.48% of ^{15}N incorporation for 10 and 20 days respectively) (WT v/s Δ*Mep2* ($p<0.005$)). However, no difference in insect derived ^{15}N incorporation relative to WT was noted in plants that were not treated with MMN for both 10 and 20 days. Δ*Urease*, showed no difference relative to WT in all the experimental

condition except 10 days in MMN treated plants. Increased ^{15}N incorporation to barley was noted in the presence of Δ*Urease* (79.93%) compared to WT (68.84%) was noted after 10 days in MMN treated plants (WT v/s Δ*Urease* ($p<0.0001$)).

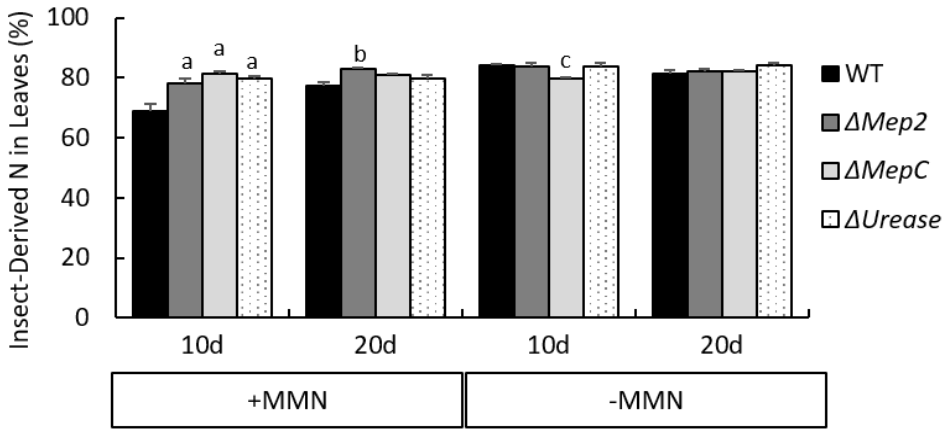


Figure 4.5 Percentage of plant nitrogen derived from ^{15}N-injected wax moth larvae by WT *Metarhizium*, Δ*Mep2*, Δ*MepC* and Δ*Urease*. Two conditions were used: Plants were treated with 25 mL of 50% MMN (+MMN) and without 50% MMN (-MMN). Amount of insect-derived nitrogen in barley leaves were determined by NOI-5 emission spectrophotometer after 10 (10d) and 20 (20d) days. The error bars represent the standard error for 6 biological replicates and the letters indicate statistically significant differences relative to WT. 'a', $p < 0.0001$; 'b', $p<0.005$; 'c', $p<0.03$.

4.4.7 Nitrogen source assay

The influence of various nitrogen sources on the growth rate (Figure 4.6) and colony morphology (Supporting Information - Figure S4.2) of ammonium permease and urease deletions was also assessed. The Δ*MepC* showed reduced colony diameter in the absence or in low concentrations of NH_4^+, relative to WT and the colony diameter for Δ*MepC* was similar regardless of the absence of nitrogen (14.66mm ±0.95), 1 mM NH_4^+ (12.91 mm ±0.49) or 30 mM NH_4^+ (13.82 mm ±0.84). Compared with WT and other gene deletion strains, Δ*MepC* showed fluffier hyphal growth in low (1 mM) and high (30 mM) NH_4^+ (Figure S4.2). In contrast, *ΔMep2* showed

a greater colony diameter relative to WT (2-way ANOVA, Tukeys's multiple comparison test: p=0.001) under no nitrogen conditions, while Δ*Urease* exhibited a colony diameter similar to WT in the absence of nitrogen. In 1 mM NH_4^+, Δ*Mep2* and Δ*Urease* showed colony diameter similar to the WT, whereas a significantly lower colony diameter relative to WT was noted for the Δ*MepC* ($p<0.0001$). A significantly greater colony diameter, compared to the WT, was observed for ammonium permease (Δ*MepC* & Δ*Mep2*) and Δ*Urease* when growth in BS agar plates supplemented with either arginine or glutamine (30mM) as the sole nitrogen source. In 1 mM proline, Δ*MepC* showed reduced colony diameter, while the colony diameter was similar to the WT in 30 mM proline. Interestingly, Δ*Mep2* exhibited a colony diameter similar to the WT in 1 mM proline, whereas a significantly higher colony diameter than WT was noted in 30 mM proline. Δ*Urease* showed less growth in BS agar medium supplemented with urea as a sole nitrogen source (Figure S4.2). No differences were observed in pH after growing in YPD and transferred to minimal media containing arginine. A slight increase in pH was noted for the WT after 6 hours in glutamine compared to the mutant strains. Additionally, Δ*Mep2* showed an increase in pH relative to WT after 6 hours and 24 hours when transferred to minimal media containing glutamate (Figure S4.3).

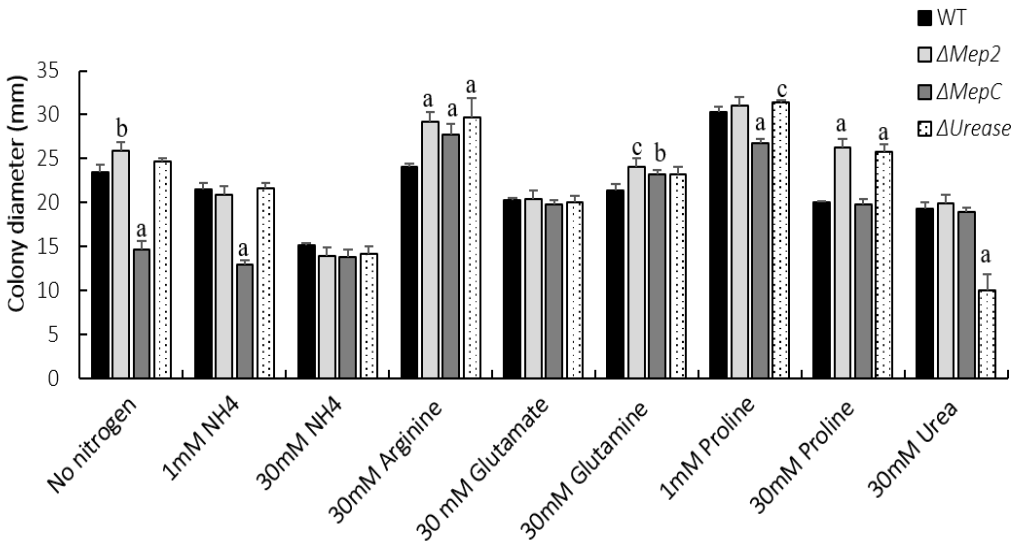


Figure 4.6 The growth rate of *M. robertsii* and mutants on BS medium supplemented with or without different nitrogen sources. The error bars represent the standard error of 5 replicates. The letters indicate significant differences relative to WT. 'a', $p < 0.001$; 'b', $p < 0.01$; 'c', $p < 0.04$.

4.5 Discussion

The deletion of two ammonium transporters in *M. robertsii*, *MepC* and *Mep2* and a sugar transporter, *Mrt*, had an influence on barley root rhizoplane association and the transfer of insect-derived nitrogen to plant hosts. The targeted deletion of a urease gene in *M. robertsii*, however showed no effect on root colonization. Surprisingly, deletions Δ*Pr1A*, Δ*Hypo. protein* and Δ*Hyd3*, that were chosen based on the high level of transcripts during root colonization showed no differences in root colonization compared to the WT.

Relative to the knowledge on the genetic attributes of insect pathogenesis, little is known about the complex relationship between *Metarhizium* and plant roots. The symbiotic interaction of *Metarhizium* with plants [179] suggests the involvement of fungal nitrogen transporters. Nitrogen

is a major nutrient requirement in plants and fungi. The two main nitrogen transporters involved in the nitrogen regulatory network in fungi are ammonium permeases and amino acid permeases [191]. Nitrogen transfer to plants during symbiosis is a common feature described in arbuscular mycorrhizal fungi which is mediated via ammonium permeases [106]. Our study showed that the targeted deletion of *MepC* or *Mep2* in *M. robertsii* resulted in enhanced rhizoplane colonization at 10 and 20 days respectively, while the colonization efficiency of Δ*Urease* was unchanged compared to the WT. The phylogenetic analysis of *M. robertsii* MepC and Mep2 amino acid sequences shown homologies to ammonium permeases in phytopathogenic, endophytic or mycorrhizal fungal species.

Ammonium and glutamine are preferred nitrogen sources for fungi. When these nitrogen sources are limited, fungi utilize other nitrogen sources. The regulatory system that enables the selective utilization of secondary nitrogen sources is known as nitrogen metabolite repression or nitrogen catabolite repression [192]. This process allows for the adaptability of fungi to changing nitrogen sources. The expression of AMT/Mep were subjected to nitrogen metabolite repression and this feature was described in non-pathogenic filamentous fungus, *Aspergillus nidulans* [193], phytopathogenic fungi (*Colletotrichum gloeosporioides*) [194] ectomycorrhizal fungi (*Hebeloma cylindrosporum*) [195]. The two GATA transcription factors identified in mediating nitrogen catabolite repression are AreA (*A. nidulans*) and nit-2 (*N. crassa*) [196]. The functionally equivalent homolog of these regulators is the nitrogen response regulator gene (*nrr1)* reported in *Metarhizium* and is suggested to play a critical role in insect virulence by regulating subtilisin-like protease and trypsin like protease expression [197]. Similar mechanisms may be operating in *Metarhizium* for plant colonization. The deletion of the ammonium permease gene may have triggered secondary nitrogen utilization pathways which subsequently increased root colonization

compared with the WT. The nitrogen source growth assays revealed the differential growth responses of Δ*Mep2* and Δ*MepC* in varying nitrogen conditions. Δ*MepC* showed reduced growth under low nitrogen conditions, while Δ*Mep2* showed no growth impairment. Previous studies demonstrated the differential expression of *mepA* and *mepB* in *A. nidulans* which were regulated by the transcription factor AreA [196]. The differential activation of ammonium transporters in response to ammonium and, consequentially, appressoria formation and virulence has been reported in the phytopathogenic fungus *C. gloeosporioides*. Their data suggested that the balance between ammonium uptake and release can induce appressorium formation by triggering signaling pathways [194]. Studies have also reported the role of signaling pathways including MAP kinase and cAMP in regulating appressorium formation and virulence in phytopathogenic [198] and entomopathogenic fungi [68, 174]. This suggests the role of signaling pathways in sensing host related stimuli (i.e. the form of nitrogen) in initiating fungal colonization on hosts. This phenomenon is not restricted to nitrogen but also to a carbon source. For example, root colonization assays of *M. robertsii* Δ*MrINV* resulted in a severe reduction of growth in root exudates, but enhanced colonization on both switch grass and *Arabidopsis* roots. The increased root colonization was suggested to be due to catabolite "derepression" in the invertase deletion mutant [99]. Interestingly, no differences in endophytic colonization were noted for the nitrogen transporter, Δ*Mrt* or the adhesin gene *ΔMad2* deletions at 10 and 20 days.

Previous studies has shown that several species of *Metarhizium* including generalist and specialist insect pathogens were able to transfer insect-derived nitrogen to plant hosts [6, 89]. The ^{15}N-transfer assay showed that, the three nitrogen transporter mutants (Δ*MepC,* Δ*Mep2* and Δ*Urease*) showed greater incorporation of insect derived ^{15}N to barley plants compared to WT when treated with 50% MMN. This suggests the adaptability of *Metarhizium* to survive in the

nitrogen rich environment in the absence of major nitrogen transporters by triggering secondary nitrogen utilization pathways. A significantly lower ^{15}N incorporation by Δ*MepC* relative to WT was observed in the absence of 50% MMN after 10 days of growth in microcosms. In contrast, the plant colonization assay revealed greater colonization by Δ*MepC* relative to WT after 10 days of association. We speculate that this variation is due to the difference in the nutrient sources available to the fungi in the rhizospheric environment that trigger different compensatory pathways dependent on nutrient availability. Previous studies reported the expression of different subset of genes by *Metarhizium* when colonizing different hosts or in different physiological conditions [71]. Furthermore, studies have shown that the pH in the rhizospheric environment can influence the amount of nitrogen absorbed by plants [199]. Previous studies demonstrated the significance of pH as an environmental cue in the host niche as a differential gene expression determinant [200].

Fang and St Leger [98], reported that disruption of the raffinose transporter gene (Δ*Mrt*) greatly reduced the rhizospheric competency of *M. robertsii* after 2 and 3 months post inoculation while no differences in rhizospheric populations were noted with WT and Δ*Mrt* after 1 week or 1 month. Consistent with this, Δ*Mrt* populations in the rhizosphere remain constant and did not significantly differ from the WT after 10 or 20 days. Surprisingly, we observed enhanced rhizoplane colonization for Δ*Mrt* strains compared to WT at 10 days similar to Δ*MepC*. In contrast, a previous study showed that Δ*Mrt* was able to colonize corn roots similar to WT but the population in the rhizospheric soil was reduced due to the inability of Δ*Mrt* to uptake oligosaccharides from root exudate [83, 98]. The cell wall protein Mad2 is a specific adhesin which enables *M. robertsii* to adhere to plant roots. Field trial experiments using corn demonstrated a reduction in survival of *ΔMad2* in rhizospheric soil as well as reduced ability to colonize plant roots [83]. However, in our study, we did not observe any differences in rhizoplane or rhizospheric association for Δ*Mad2*

compared to the WT. This inconsistency in Δ*Mrt* and Δ*Mad2* with regard to root colonization ability could be probably due to the difference in the fungal inoculation method (drench method around planted seedlings) as well as the substrate (vermiculite) we employed for planting, while *Metarhizium* treated corn seeds were used for the field trial study [83].

The targeted deletion of genes (*Pr1A, Hyd3* and *Hypo. protein*) that were upregulated in plant roots (RNA-Seq data) had no impact on endophytic, rhizoplane or rhizospheric colonization. The deletion of *subtilisin-like serine protease* (*Pr1A*) did not affect root colonization ability of *M. robertsii*. Pr1A is the predominant protease upregulated during *Metarhizium* infection of the insect cuticle [72] and is an important gene used for strain improvement for biocontrol purposes. The integration of multiple copies of *Pr1A* and its constitutive expression in *M. anisopliae* increased virulence against insect hosts [201]. The expression of subtilisin-like protease has been reported in a plant pathogenic fungus, *Magnaporthe poae*, a pathogen of Kentucky bluegrass. The expression of the protease increased on infected roots [202]. Similar subtilisin-like proteases have also been demonstrated in grass endophytic fungi including *Neotyphodium typhinum* [73] and *Epichloe festucae* [203], however, the specific role of these proteases during plant colonization is unknown. Greater expression levels of *Pr1A* has been demonstrated in *M. robertsii* grown in bean root exudate [81], however, evidence suggests that the expression of *Pr1A* is correlated to a stress response and nutrient availability. Hence, the expression of *Pr1A* is proposed as a stress response by *M. robertsii* under nutrient starvation conditions including bean root exudate [71] and possibly during plant root colonization. Moreover, previous studies suggested the evolutionary role of different proteases that resulted from gene duplication, loss and horizontal gene transfer events, is correlated with the multiple life styles of *M. robertsii* [11].

The role of hydrophobins during initial interaction and virulence with plant hosts have been investigated in phytopathogenic and endophytic fungi [105, 159, 160, 169, 187]. Although *Hyd3* was upregulated in *Metarhizium*-bean root transcriptome, the disruption of *Hyd3* did not affect the rhizoplane or rhizospheric colonization ability of *M. robertsii*. Sequence analysis revealed 96% sequence similarity with *M. brunneum HYD3*. Comparably, the loss of the *Hyd3* gene did not affect plant rhizosphere competency for *M. brunneum* (unpublished data). Furthermore, previous studies have also revealed the contribution of hydrophobins in the entomopathogenic/endophytic fungus *B. bassiana* in root colonization of *Phaseolus vulgaris* [187]. The consequences of the targeted deletion of hydrophobin genes on fungal development and interaction with hosts can vary in different fungi. Besides, the presence or upregulation of other hydrophobins or signaling pathways could compensate or mask homologous gene deletions.

cDNA microarray analysis in *M. anisopliae* have shown the differential expression of a large number of hypothetical proteins in insect cuticle (26.6%), insect hemolymph (22.7%) and bean root exudate (29.9%) [71]. Similarly, a large number (41.4%) of the upregulated genes in *M. anisopliae* when grown in root exudate coded for hypothetical/unknown proteins [81]. In our study, the transcriptome analysis of *Metarhizium*-colonized bean roots showed that 25.81% of the upregulated genes (10-234 reading) coded for hypothetical proteins. *MAA_08959* (*Hypo. protein*) showed the highest expression among these and the root colonization assays showed that the targeted deletion of MAA_08959 (*Hypo. protein*) did not affect the root colonization ability of *M. robertsii*. This indicates a potentially significant role for uncharacterized proteins in the multiple lifestyles of *M. robertsii*.

Nitrogen source assays showed that Δ*MepC* and Δ*Mep2* grew differently depending on nitrogen sources. *Mep2* appears to play a minor role in nitrogen metabolism since the targeted

deletion of *Mep2* did not inhibit its growth rate in nitrogen starvation conditions. This indicated that the loss of *Mep2* might be fully compensated by the expression of *MepC*. Similarly, the targeted deletion of *MepA* in *Fusarium fujikori* did not affect the growth rate of this fungus, and the gene deletion was completely compensated by the expression of other *Mep* genes [192]. The targeted deletion of *MepC* resulted in starvation induced growth inhibition in *M. robertsii*, while the loss of *Mep2* resulted in increased growth rate during nitrogen starvation. This data corresponds to the phenotype described for *Aspergillus nidulans* Δ*MeaA* and *F. fujikori* Δ*MepC* mutants, where reduced growth rates were observed during growth in low ammonium concentrations [192, 193].

M. robertsii can infect and kill a wide range of insect species [12]. The molecular and the biochemical factors involved in insect pathogenicity is well studied [3, 47]. Disruption of *MepC*, *Mep2*, *urease* or *Hypo. protein* gene did not alter the virulence of *M. robertsii* against mealworm or wax-moth larvae. However, the disruption of the *Hyd3 or Pr1A* resulted in delayed mortality compared to the WT but only against mealworm larvae. The deletion of *Hyd3*, which encodes a Class I hydrophobin, resulted in altered hydrophobicity and conidiation in *M. robertsii*. Consistent with this, the deletion of *Hyd3* affected conidiation, surface hydrophobicity and pathogenicity in *M. brunneum* [9]. In *B. bassiana*, two Class I hydrophobins, hyd1 and hyd2 were reported to play distinct roles in fungal development and interaction with insect hosts. The targeted deletion of *hyd1* altered rodlets on the surface of conidia, lowered surface hydrophobicity and virulence, but the conidia retained adhesion qualities. While the deletion of *hyd2* resulted in decreased surface hydrophobicity and adhesion phenotype, the virulence towards the insect host was not affected [10]. Subtilisin-like serine proteases have been reported in nematode-parasitic, mycoparasitic and entomopathogenic fungi as a virulence factor [74, 77]. Subtilisin-like proteases can degrade the protein linkages present on the host integument and thus can mediate the penetration and further

colonization events during infection. The role of *M. robertsii* Pr1A during insect pathogenesis has been reported previously [204].

In conclusion, although high levels of expression of *Pr1A*, *Hypo. protein*, and *Hyd3* was observed in bean root, the disruption of these genes did not affect the root or rhizosphere colonization ability of the fungus. The deletion of hypothetical protein gene had little impact on insect virulence, while *Pr1A* and *Hyd3* contributed to pathogenicity against meal worm. The expression of Hyd3 plays a significant role in conidial hydrophobicity in *M. robertsii*. The impact of gene deletions depends on several features including compensatory gene expression or the plasticity adapted by the organism in order to survive in that environment. The effect of synthetic gene alterations may be effective only under certain environmental or nutrient conditions where the organism is limited in its ability to express compensatory genes [205]. Furthermore, we report the involvement of ammonium permease genes (*MepC* and *Mep2*) of *M. robertsii* during plant root colonization as well as in insect derived nitrogen transfer. Nitrogen transporters, *MepC*, *Mep2* and *urease* have no role in insect pathogenicity which was confirmed in two insect hosts. These genes play a critical role in the growth of fungi in nitrogen rich and low conditions. However, a deeper insight into the underlying processes that results the stable association between *M. robertsii* and plant host are necessary. The identification and characterization of symbiosis related genes and specific functions will provide a deeper understanding on plant root colonization formed between this ecologically and agronomically important fungus and different plant hosts.

4.6 Supporting Information

Table S4.1. Primer pairs used in the study.

Primer	Sequence (5'-3')	Function
MAA_04182-5-1	ggTCTAGACCTGACGCACGATGTAG	
MAA_04182-5-2	ggGAATTCTAGCAGGCATCGGAGTA	Disruption of *MAA_04182*
MAA_04182-3-1	ggACTAGTTGCTTGTTATCCCGTCT	
MAA_04182-3-2	ggCCCGGGCCCTTTGGCTTCTGACT	
MAA_04182-CF1	TCATCGTTTGTGCCATT	Confirmation of the disruption of
MAA_04182-CF2	TGGTTGTCTCCGTGGTG	*MAA_04182*
MAA_05002-5-1	ggTCTAGATTTCTGGCAGTATGGTC	
MAA_05002-5-2	ggACTAGTATACTTTGCCAAGGTTC	Disruption of *MAA_05002*
MAA_05002-3-1	ggACTAGTGACACTGGCAGGGTTGT	
MAA_05002-3-2	ggCCCGGGAGCAGACGGGAGCTTAG	
MAA_05002-CF1	TTCCATTGTATTCAGCC	Confirmation of the disruption of
MAA_05002-CF2	AATCCTGTTACAGACCG	*MAA_05002*
MAA_07458-5-1	ggTCTAGACCTCTATCAAGGATGAG	
MAA_07458-5-2	ggGAATTCTATTAGATAGCAACGGA	Disruption of *MAA_07458*
MAA_07458-3-1	ggACTAGTAAGAAGGAAACCAAGAA	
MAA_07458-3-2	ggGATATCAGTAAGGAGCGGTGTAT	
MAA_07458-CF1	GCTTCCGAGTGATTTAG	Confirmation of the disruption of
MAA_07458-CF2	AAGGCTCGCAGAGGTTC	*MAA_07458*
MAA_05675-5-1	ggACTAGTCGCTAGGCTCTACATTG	
MAA_05675-5-2	ggACTAGT TGAATCCAGGACAGATC	Disruption of *MAA_05675*
MAA_05675-3-1	ggTCTAGATTTCCAACGCTCGAATC	
MAA_05675-3-2	ggCCCGGGGGAACGGGTTGTTTGAG	
MAA_05675-CF1	CAACGGTGCCTAAATTC	Confirmation of the disruption of
MAA_05675-CF2	ACGTGTTGGCTAGAATG	*MAA_05675*
MAA_08959-5-1	ggGCTAGCTCCTTGTGTGATCGTTG	
MAA_08959-5-2	ggGAATTCCGTGTCCAAGTATAGAG	Disruption of *MAA_08959*
MAA_08959-3-1	ggGCTAGCCTAGGTCTCGAATACAG	
MAA_08959-3-2	ggGATATCTGCAGCCTTACATTGTC	
MAA_08959-CF1	TGATGAAGACGTGGTTG	Confirmation of the disruption of
MAA_08959-CF2	ATAGCGGTTCGACCAAG	*MAA_08959*
MAA_10298-5-1	ggTCTAGACGGATAAGATTCGAGTC	
MAA_10298-5-2	ggGAATTCTTGTGAGTGAGAGTGAG	Disruption of *MAA_10298*
MAA_10298-3-1	ggTCTAGAGCTAGTCAGGTGGTTTG	
MAA_10298-3-2	ggCCCGGGGCTGGTTGGTGTACTTG	

Table S4.1 – contd.

Primer	Sequence (5'-3')	Function
MAA_10298-CF1	GCAACCCATGGTTGTAC	Confirmation of the disruption of *MAA_10298*
MAA_10298-CF2	CTCTTGAGTACGGTAAG	
Bar-up	CGCCTGGACGACTAAACC	Confirmation of the disruptions
Bar-down	TCAGCCTGCCGGTACCGC	

* CF2/bar to verify the correct integration of bar gene in target gene. CF1/CF2 to verify the WT and absence of target gene in mutant.

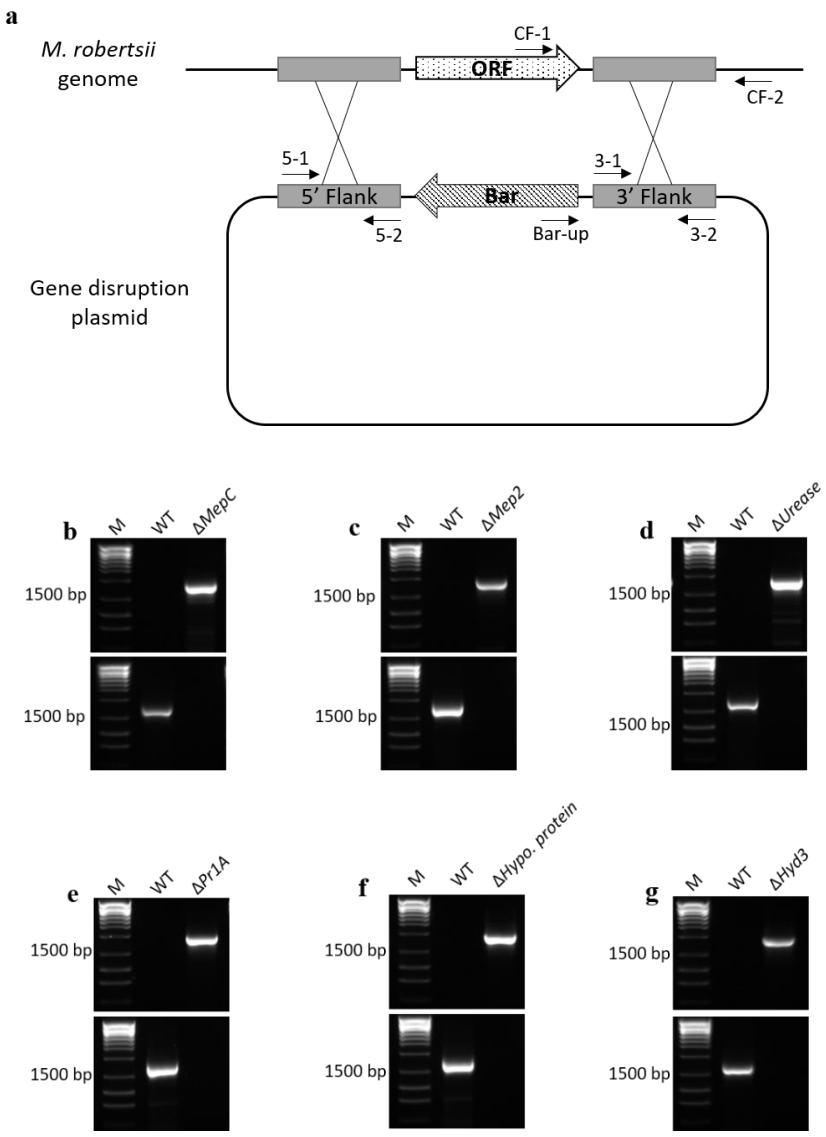


Figure S4.1 a. Schematic representation of construction of targeted gene deletion mutants based on homologous recombination and showing a map of a disruption plasmid and its relative position in the *Metarhizium* genome. The herbicide resistance gene (bar) were inserted in to the open reading frame (ORF) of the target gene. **b-g.** PCR verification of correct integration event in mutants. **b.** Confirmation of construction of *MepC* deletion mutant. **c.** Confirmation of construction of *Mep2* deletion mutant. **d.** Confirmation of construction of *Urease* deletion mutant. **e.** Confirmation of construction of *Pr1A* deletion mutant. **f.** Confirmation of construction of *Hypo. protein* deletion mutant. **g.** Confirmation of construction of *Hyd3* deletion mutant. The top panel of b, c, d, e, f and g: The PCR conducted with primers bar-up/bar-down and confirmation primer CF-2; The PCR products can be obtained only for deletion mutants of each gene not for the wild type (WT). The bottom panel of b, c, d, e, f and g: The PCR conducted with confirmation primers CF1and CF2. PCR products can be obtained only for WT and not for deletion mutants. M – DNA ladder.

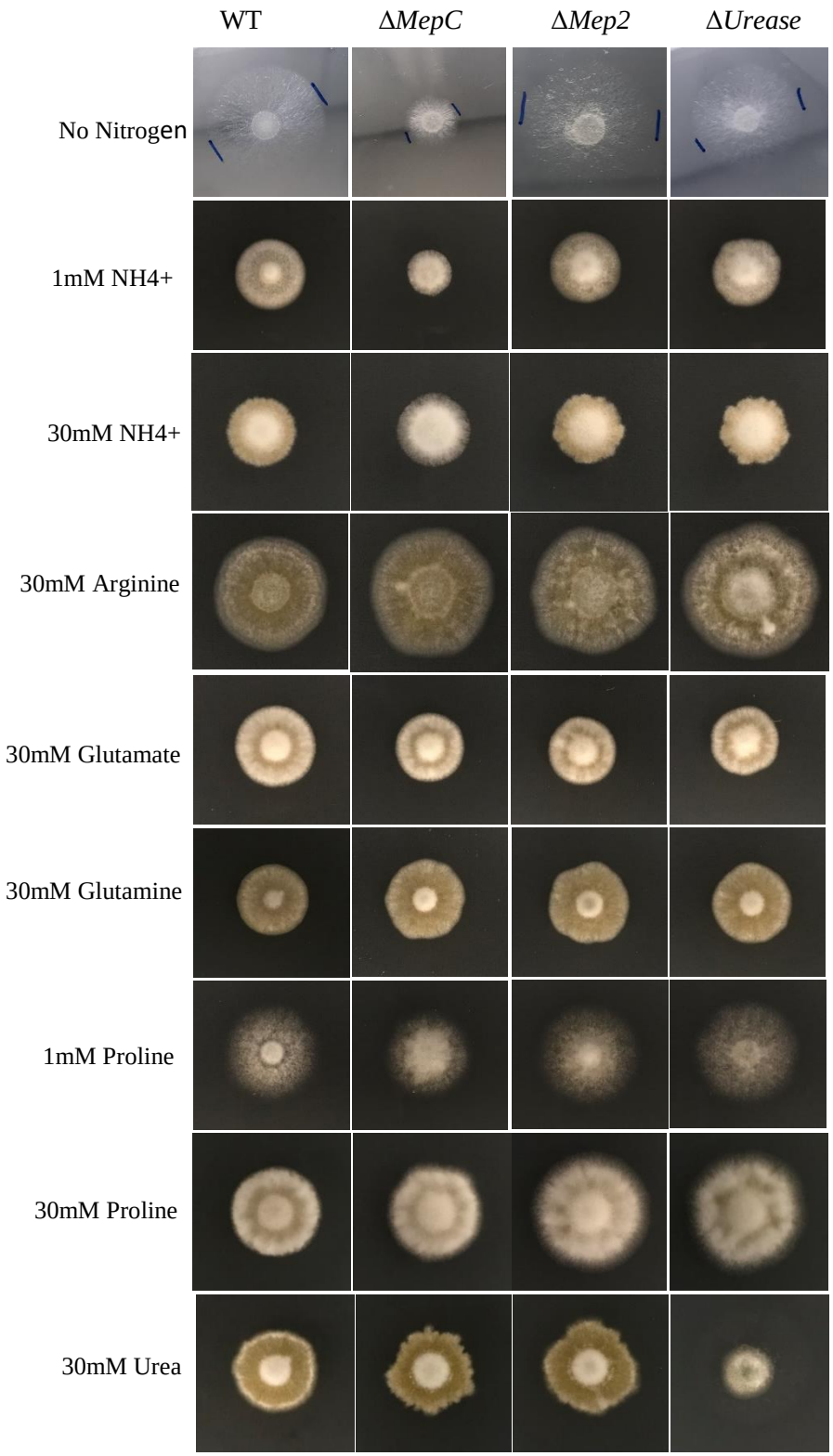


Figure S4.2. The colony morphology of WT and mutant strains grown in BS media supplemented with or without different nitrogen sources.

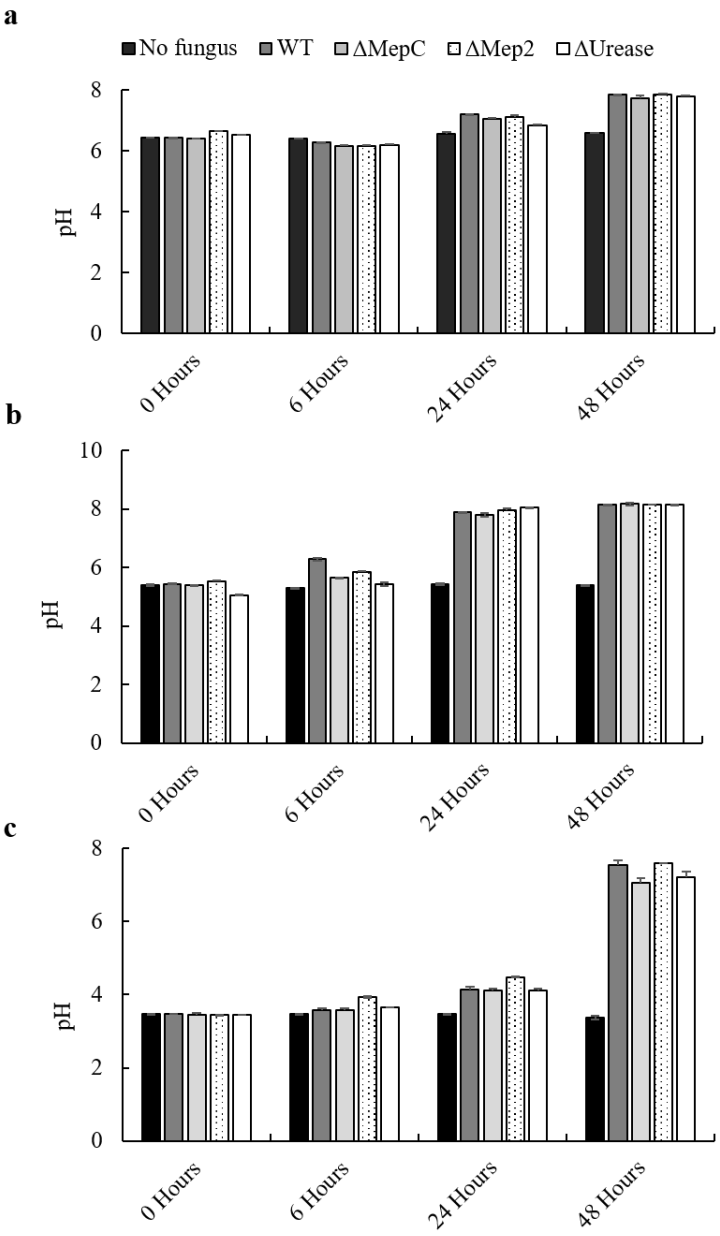


Figure S4.3. The ammonia production based on pH of the mutant strains and WT. 1 mL of the conidial suspension was added on to potato dextrose broth and allowed to grow for 4 days. Mycelia was then filtered, washed with sterile distilled water and transferred to minimal media supplemented with different amino acids. 2.5 g of filtered fungal mycelia was added to 100 mL of minimal media broth and incubated at 100 rpm at 27ºC. The samples were collected from the cultures was collected at regular intervals (0, 6, 24 and 48 hours) to check the pH. **a**. Arginine, **b**. Glutamine, **c.** Glutamate.

Chapter 5 - Generalist and specialist *Metarhizium* insect pathogens retain ancestral ability to colonize plant roots

Authors: Soumya Moonjely and Michael J. Bidochka

5.1 Abstract

Metarhizium is widely recognized as an insect pathogenic fungus but it can also form symbiotic associations with plant roots. Here we assessed root rhizoplane, as well as endophytic colonization in monocots and dicots by ten *Metarhizium* strains representing species with either narrow or broad insect host ranges, as well as a related nematophagous fungus, *Pochonia chlamydosporia*. In addition, insect pathogenicity of these strains was evaluated against meal worm, wax moth larvae and grasshoppers. We found that all *Metarhizium* strains and *Pochonia* colonized the rhizoplane and rhizosphere of roots with varying degrees on all plants assessed. All *Metarhizium* strains tested showed a preference for endophytic colonization on monocot plants within 20 days when compared to dicots. While generalists showed pathogenicity towards all the insect hosts tested, the specialists showed pathogenicity only towards grasshoppers, whereas *Pochonia* was avirulent towards insects. We observed variation in ability to colonize the rhizosphere amongst *Metarhizium* species. However, regardless of whether the *Metarhizium* species was a generalist or specialist insect pathogen all strains tested retain ancestral ability to associate with plant roots.

5.2 Introduction

Species in the ascomycetous genus *Metarhizium*, are generally referred to as facultative insect pathogenic fungus and have been used as insect biocontrol agents [206]. *Metarhizium* spp.

have a versatile lifestyle and can persist in diverse habitats as saprophytes [52], insect pathogens [111] or as rhizosphere colonizers [25]. Phylogenomic studies suggest that *Metarhizium* diverged from Clavicipitacean endophytes (*Epichloe* and *Claviceps*) approximately 100 MYA (Gao et al., 2011). Insect pathogenicity exhibited by *Metarhizium* is a more recently acquired trait which may have been derived from *Metarhizium* genes involved in plant colonization or possibly acquired from prokaryotes via, horizontal gene transfer [87]. *Metarhizium* is an extremely diverse genus with more than 30 different species [207]. Some *Metarhizium* species have broad insect host range while others have a narrow insect host range. The divergence between generalist and specialist strains occurred ca. 35 MYA [11]. For example, *M. robertsii* is a generalist and can infect insects in the orders Orthoptera, Dermaptera, Hemiptera, Diptera, Hymenoptera, Lepidoptera, and Coleoptera; whereas, *M. acridum* is a specialist with a narrow insect host range restricted to Orthoptera (grasshoppers, locusts or crickets) [14]. *Metarhizium* speciation shows divergence from specialist (*M. acridum*) with narrow host ranges to transitional species with intermediate host ranges (*M. guizhouense*) followed by generalist species (*M. robertsii*). Besides the insect host range, differences in genome sizes and protein families have also been observed and are larger in generalist species than in specialists. Furthermore, specialist species have fewer metabolite producing genes than generalist species or in other plant associated fungi [87].

Although *Metarhizium* spp. are adapted as insect pathogens, many of the species associate with the plant rhizosphere. *Metarhizium* spp. have been isolated from certain plants [94] and particularly from plant roots [79]. Moreover, the prevalence of certain *Metarhizium* species in a specific geographical area or associations with certain plants have been reported [208–211]. For example, *M. brunneum* was found to preferentially colonize blue berries and strawberries while *M. guizhouense* and *M. robertsii* show significant association with coniferous trees in Oregon,

USA [212]. Previous studies demonstrated that *Metarhizium* species showed an association to a specific habitat type rather than insect hosts [213]. Field studies conducted with a recombinant *Metarhizium* have shown increased persistence of fungal propagules in rhizospheric soil of cabbage rather than in bulk soil. The rhizospheric microenvironment seems to have an influence in the persistence of *Metarhizium* in soil [52]. Numerous studies have demonstrated the plant colonizing ability of different *Metarhizium* spp., particularly *M. anisopliae*, *M. brunneum*, *M. robertsii* [6, 83]. An increase in plant growth was reported when corn and bean soils were treated with *Metarhizium*. *M. robertsii* can colonize switch grass, haricot bean, wheat and soy bean as a root symbiont and can transfer insect-derived nitrogen from the infected insect host to their plant symbionts [6] in exchange for carbon [179]. Most of the studies that evaluated *Metarhizium* plant association were focused on elucidating the diversity and abundance of *Metarhizium* species in a selected habitat. Little is known of the range of root colonization by specialist and generalist insect pathogen species or the colonization preferences toward any particular plant species. To this end, we investigated the root colonizing (rhizospheric and endophytic) abilities of different *Metarhizium* spp. on monocot and dicot plant species under greenhouse conditions.

The initial adhesion of the fungal conidia to host surfaces is critical for the successful establishment of parasitic, symbiotic or mutualistic relationship with hosts. In most fungus, this is mainly mediated via, non-specific interactions which is achieved by cell wall surface proteins called hydrophobins. Hydrophobins are amphipathic proteins unique to filamentous fungi that impart hydrophobicity to fungal conidia [7, 214, 215]. The role of hydrophobins have been reported in other entomopathogenic [9], endophytic [187] and plant pathogenic fungi [105, 157, 159, 169]. Gene disruption studies in another endophytic insect pathogenic fungus, *Beauveria bassiana*, have shown that hydrophobins play a role in conidial surface properties and virulence

towards insect hosts. Moreover, the deletion of *hyd* genes affected the root colonization ability of the fungus [10, 187]. However, variation in conidial hydrophobicities and its implication in insect or plant interaction have not been compared in different *Metarhizium* species.

In this study we tested the abilities of generalist and specialist *Metarhizium* spp. to form rhizospheric associations in monocots and dicots. We also included assays with the closely related endophytic fungus *P. chlamydosporia.* In addition, we verified host specificity towards three insects (wax moth larvae, meal worm and grasshopper). Furthermore, growth rates and the variation in the conidial hydrophobicity among *Metarhizium* strains was assessed.

5. 3 Materials and methods

5.3.1 Fungal isolates and plant material

Ten strains of *Metarhizium* species were used in this study; *Metarhizium robertsii* (2575), *M. acridum* (7486), *M. flavoviride* (380189), *M. brunneum* (KTU60 and 432ai), *M. pingshaense* (MP1), *M. guizhouense* (B77-ai) and, kindly provided by Brian Lovett, University of Maryland, *M. frigidum* (4124), *M. anisopliae* (549), *M. pingshaense* (S10). *P. chlamydosporia* (isolated from an ant hill was included in the assays [216]. Fungal cultures were routinely grown and maintained on Potato Dextrose Agar (PDA, Bioshop Canada Inc., Burlington, ON) at 27^{o}C for 14 days, and conidial suspensions were prepared (10^7 conidia/mL) in 0.01% Triton X-100. Colony morphologies and growth rates were examined by inoculating 10 µL of a $1x10^7$ conidia/mL suspension into the center of a PDA (10 mL) plate. The colony diameters were measured at 3, 7, 14 and 21 days.

Phaseolus vulgaris (haricot bean) and *Zea mays* (maize or corn) seeds were obtained from OSC seeds (Kitchener, ON). *Hordeum vulgare* (barley) seeds were obtained from Sprout Master (Elmvale, ON). *Capsicum annum* (green bell pepper), *Pisum sativum* (peas) and *Solanum lycopersicum* (tomato) seeds were obtained from Stokes Seeds Ltd (Thorold, ON).

5.3.2 Root colonization assays

The endophytic, rhizoplane and rhizospheric association of *Metarhizium* isolates and *P. chlamydosporia* were analyzed. Haricot bean, barley, pepper, peas and tomato seeds were surface sterilized with 4% sodium hypochlorite (NaOCl) three times for 5 minutes. The seeds were rinsed with sterile distilled water after each NaOCl wash. After sterilization, seeds were kept overnight at 4^{o}C for stratification of germination before planting. The seeds were then allowed to germinate in water agar or sterile vermiculite for 3-4 days at 25^{o}C. The corn seeds were soaked in distilled water for 3-4 hours before sterilization. The seeds were then surface sterilized with 4% sodium hypochlorite for 20 minutes and subsequently rinsed in sterile distilled water to remove all traces of NaClO. The corn seeds were then allowed to germinate in autoclaved vermiculite for 3-4 days at 25^{0}C.

The germinated seedlings were then planted in autoclaved vermiculite (Ther-O-Rock East Inc., New Eagle, PA). Fungal inoculations were performed using the drench method [186] where the conidial suspensions (5 mL) were poured onto the surface of vermiculite for each pot. The plants were kept in a greenhouse at 25^{o}C during the day with photoperiod of 16:8 h light: dark cycle with relative humidity maintained between 60-80%. The plants were watered daily with sterile distilled water. Five biological replicates were prepared for each treatment. To quantify fungal association, the roots were harvested from 10 and 20 day old plants. The amount of fungal association on harvested roots was analyzed as described previously [187]. To examine the

endophytic association, the harvested roots were first washed in water to remove the attached vermiculite. The washed roots were then immersed in 2% NaOCl for 10 seconds and finally rinsed with sterile distilled water to remove the traces of NaOCl. The roots were then cut into ~2-5 mm pieces, weighed and homogenized (Biospec products Inc., Bartlesville, OK) in sterile distilled water for 2 mins. The homogenized root samples were then plated onto modified CTC agar (PDA supplemented with 0.5 g/L chloramphenicol, 0.004 g/L thiabendazole and 0.5 g/L cycloheximide) [166] and CFU values were calculated as CFU/g of root weight. To assess rhizoplane colonization, the harvested roots were treated as described above except that the surface sterilization with 2% NaOCl was omitted. Water inoculated plants were treated as controls. Rhizospheric populations of *Metarhizium* and *Pochonia* were also monitored. Here, the vermiculite attached to the roots was collected during the harvest, weighed and suspended in 0.01% Triton X-100. The serial dilutions of the suspension were plated on CTC media and the CFU were calculated.

5.3.3 Insect bioassays

The virulence of *Metarhizium* isolates and *P. chlamydosporia* was assayed against *Melanoplus sanguinipes* (migratory grasshopper, non-diapause colony*) (*Agriculture & Agri-Food Canada Saskatoon Research Centre, Saskatoon, SK), *Tenebrio molitor* larvae (meal worm) (Pet Smart, St. Catharines, ON) *and Galleria mellonella* larvae (wax moth) (Massasauga Imports, Acton, ON).

In order to infect insects, 10 µL of a conidial suspension ($1x10^7$ conidia/mL) were applied to the cuticle (abdominal segments) of the larvae or the dorsal pronotum for the adult grasshoppers. Each insect was placed separately in a 60mm x 15mm diameter Petri dish. Humidity was maintained in each Petri dish with a moistened piece of filter paper. Lettuce was provided to the grasshoppers *ad libitum*. The treated insects were then placed at 25^{o}C and mortality was recorded

daily. Each replicate contained 20 larvae and was performed in duplicate. The carrier, 0.01% Triton X-100, was used as the negative control. The LT50 values were calculated using Probit analysis.

5.3.4 Conidial hydrophobicity assay

Conidial hydrophobicity was assessed as previously described [10]. Briefly, conidia were harvested from 14 day old PDA plates by flooding with 0.01% Triton X-100. The harvested conidia were then washed in sterile distilled water and subsequently resuspended in reaction buffer (22.2 g K_2PO_4, 7.26 g KH_2PO_4, 1.8 g urea, 0.2 g $MgSO_4$ per L, pH 7.1). The concentration of the conidial suspension was adjusted to OD_{470} = 0.4. The conidial suspension (3 mL) was then transferred to glass vials to which 300 µL of hexadecane was added. The tubes were vortexed for 30 seconds, 3 times and kept at room temperature for 15 minutes. The hexadecane phase was removed from each tube. The residual solidified hexadecane was removed after incubating the tubes at 5^oC for 10 minutes. The tubes were then transferred to room temperature and the absorbance of the suspensions was determined at A_{470} using a spectrophotometer. The hydrophobic index of each conidial suspension were then calculated using the given equation ($A_{470\ control}$ - $A_{470\ hexadecane\ treated}$)/($A_{470\ control}$).

The adhesion of conidia to a hydrophobic surface was also analyzed. The conidial concentration was adjusted to $1x10^6$ conidial/mL in sterile distilled water and 100 µL of the conidial suspension was applied onto the hydrophobic surface of gel bond film. After 3 hours the surface was washed with PBS (phosphate buffered saline, pH 7.4) buffer and conidia attached to the hydrophobic surface were counted.

5.4 Results

5.4.1 Colony morphologies and growth rates

Colony morphologies of *Metarhizium* spp. and *Pochonia* in PDA are shown in Figure 5.1. The differences in growth rates among different *Metarhizium* spp. were observed (Figure 5.2). *M. frigidum* exhibited white fluffy mycelia and a lower growth rate relative to other *Metarhizium* strains. After 21 days the colony diameter was 19.35 mm (±1.17) while the colony diameters of other *Metarhizium* strains ranged from 48-75 mm. The two *M. brunneum* strains (KTU 60 and 432ai) showed yellow colony morphology and a significantly slower growth rates when compared to *M. anisopliae, M. flavoviride*, *M. robertsii*. *M. guizhouense* appeared as white thick fluffy cottony mycelia while *M. anisopliae* and *M. pingshaense* exhibited flat and fibrous white mycelia after 7 days of growth. *P. chlamydosporia* showed pink powdery colony morphology and a higher growth rate relative to all *Metarhizium* strains.

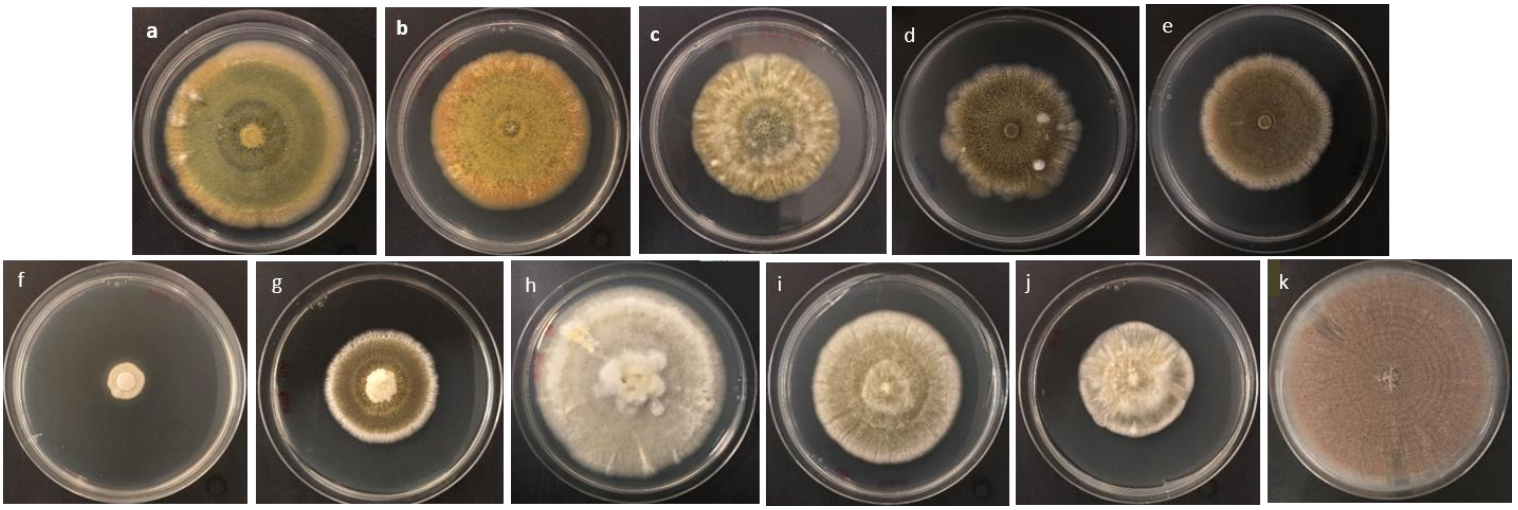


Figure 5.1 Colony morphologies of fungal isolates used in the study. a. *M. acridum* (7486), **b.** *M. flavoviride* (380189), **c.** *M. brunneum (KTU60),* **d.** *M. brunneum* (432ai), **e.** *M. anisopliae* (549), **f.** *M. frigidum* (4124), **g.** *M. guizhouense (B77-ai),* **h.** *M. robertsii* (2575), **i.** *M. pingshaense* (MP1 isolated from soil), **j.** *M. pingshaense* (S10), **k.** *Pochonia chlamydosporia* (isolated from ant-hill). The images shown are from cultures grown on PDA at 27^0C in the dark for 21 days.

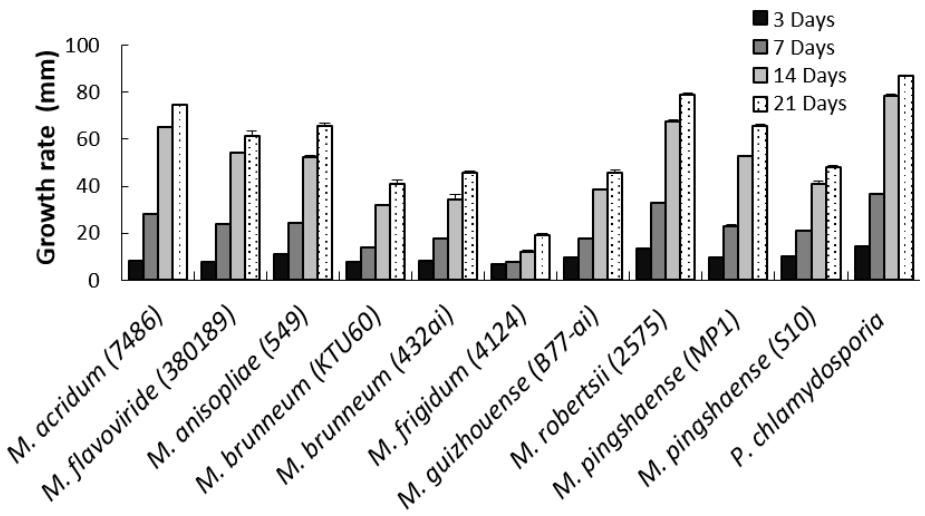


Figure 5.2 Growth rates of *Metarhizium* strains and *Pochonia* on PDA. A conidial suspension of 10 µL ($1x10^7$ conidia/mL) was inoculated on PDA plates and the colony diameter was measured after 3, 7, 14 and 21 days. The error bars represent the standard error of five biological replicates.

5.4.2 Interaction with dicot and monocot roots by fungal isolates

Metarhizium strains and *P. chlamydosporia* used in this study formed rhizospheric and rhizoplane associations with the six plant species used in this study. However, endophytic colonization was only observed in monocots during the first 20 days with the exception of pepper plants, the only dicot where endophytic colonization was observed after 20 days. All values are in CFU/g of root for endophytic or rhizoplane colonization and CFU/g of vermiculite for rhizospheric colonization.

5.4.2.1 Endophytic colonization: Endophytic colonization was not observed in dicot plants including beans, peas and tomato for ten *Metarhizium* strains with the exception of pepper. All values are in CFU/g of root. In pepper, *M. acridum*, *M. brunneum* (KTU60 and 432ai), *M.*

frigidum, *M. pingshaense* (S10), showed relatively low levels ($1.2x10^2$ - $3.4x10^2$) of endophytic colonization in comparison to *P. chlamydosporia* ($1.3 x10^4$) after 20 days. However, in the monocots, barley and corn, all *Metarhizium* species showed endophytic colonization (Figure 5.3a and b). In barley, no differences in endophytic colonization were observed among *Metarhizium* strains after 10 days. *P. chlamydosporia* showed higher endophytic colonization ($1.6 x10^4$) on barley roots compared to all *Metarhizium* strains (Tukey Honestly Significant Difference (Tukey HSD), $p<0.0009$) at 10 days. At 20 days, *M. flavoviride* ($9.8x10^3$) showed ($p<0.05$) higher endophytic colonization compared to *M. pingshaense* (MP1) ($2.7 x10^3$), *M. pingshaense* (S10) ($1.2 x10^3$), *M. acridum* (7486) ($3.9 x10^2$), *M. anisopliae* (549) ($1.3 x10^3$), *M. brunneum* (KTU60) ($1.6 x10^3$), *M. frigidum* (4124) ($2.2 x10^3$). Of the 10 *Metarhizium* strains used, *M. acridum* showed lowest endophytic colonization ($3.9 x10^2$) on barley roots.

On corn roots (Figure 5.3b), *M. pingshaense* (S10) ($5.1x10^3$) showed higher ($p<0.04$) endophytic colonization after 10 days relative to other *Metarhizium* strains except for *M. frigidum* ($3.5x10^3$). After 20 days, no significant differences in the endophytic colonization were observed amongst *Metarhizium* strains. However, *P. chlamydosporia* exhibited higher endophytic colonization ($p<0.00009$) relative to *Metarhizium* strains after 10 or 20 days.

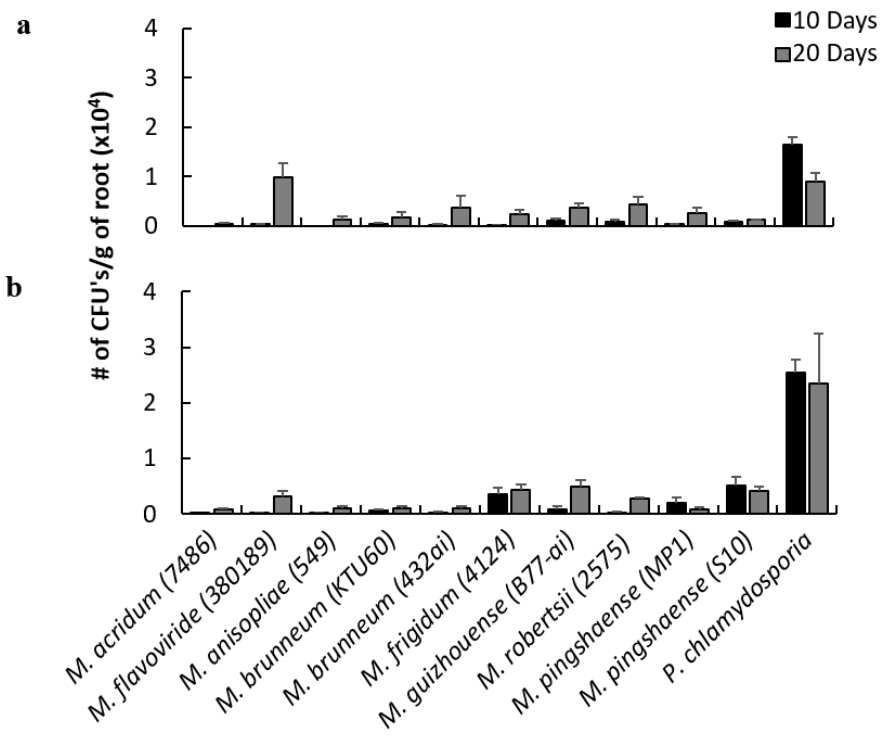


Figure 5.3 Endophytic colonization of fungal strains. **a.** Barley, **b.** Corn after 10 and 20 days. The error bars represent the standard error for five biological replicates.

5.4.2.2 Rhizoplane colonization: All species of *Metarhizium* and *P. chlamydosporia* were able to form rhizoplane associations with corn, barley, haricot beans, peas, pepper, and tomato roots after 10 and 20 days (Figure 5.4). All values are in CFU/g of root.

After 10 days post inoculation on barley and corn *P. chlamydosporia* showed greater ($3.47x10^5$ and $3.47x10^5$, respectively) rhizoplane association relative to the *Metarhizium* strains (Figure 5.4a and b). Amongst the *Metarhizium* strains, no differences in rhizoplane associations were observed after 10 day on barley and corn roots. However, higher rhizoplane colonization was noted for *M. flavoviride* ($1.81x10^5$, $p<0.004$) relative to *M. pingshaense* (S10 and MP1), *M. robertsii*, *M. acridum*, *M. anisopliae*, *M. brunneum* (KTU60), *M. guizhouense* after 20 days on

barley. *M. frigidum* also exhibited comparable greater rhizoplane association ($1.81x10^5$) on barley roots at 20 days. Unlike barley, none of the *Metarhizium* strains showed difference in rhizoplane association after 20 days on corn roots.

In haricot beans (Figure 5.4c), *M. acridum* showed lower ($p<0.01$) rhizoplane colonization compared to *M. brunneum, M. robertsii, M. guizhouense, P. chlamydosporia.* Except for *M. brunneum* (432ai), all other *Metarhizium* strains showed significantly lower rhizoplane interaction ($p<0.004$) on bean roots after 10 days. No differences in rhizoplane interaction was observed for *Metarhizium* strains compared with *P. chlamydosporia* after 20 days. Similar to the 10 day post treatment, *M. brunneum* exhibited comparably higher rhizoplane association on bean roots when compared with other *Metarhizium* strains.

In peas, after 10 days post treatment (Figure 5.4d), no differences in rhizoplane colonization were observed among the 11 fungal strains. However, after 20 days a decrease in rhizoplane colonization was noted for *M. pingshaense* (S10) ($p<0.03$) relative to *M. frigidum, M. guizhouense* and *P. chlamydosporia*. Amongst the *Metarhizium* strains, a comparably higher CFU was observed for *M. guizhouense* ($3.12x10^4$) ($p<0.01$) compared with *M. acridum* ($1.24x10^3$), *M. anisopliae* ($1.10x10^4$), *M. brunneum* (KTU60), *M. brunneum* (432ai), *M. pingshaense* (S10) after 20 days. Surprisingly, except for *M. acridum, M. brunneum* (KTU60), *M. pingshaense* (S10), no other *Metarhizium* strains showed a difference in rhizoplane association compared to *P. chlamydosporia* after 20 days.

For tomato and pepper, an increase in CFU was observed for *P. chlamydosporia* after 10 and 20 days when compared with all *Metarhizium* species. However, no differences were observed among *Metarhizium* strains (data not shown).

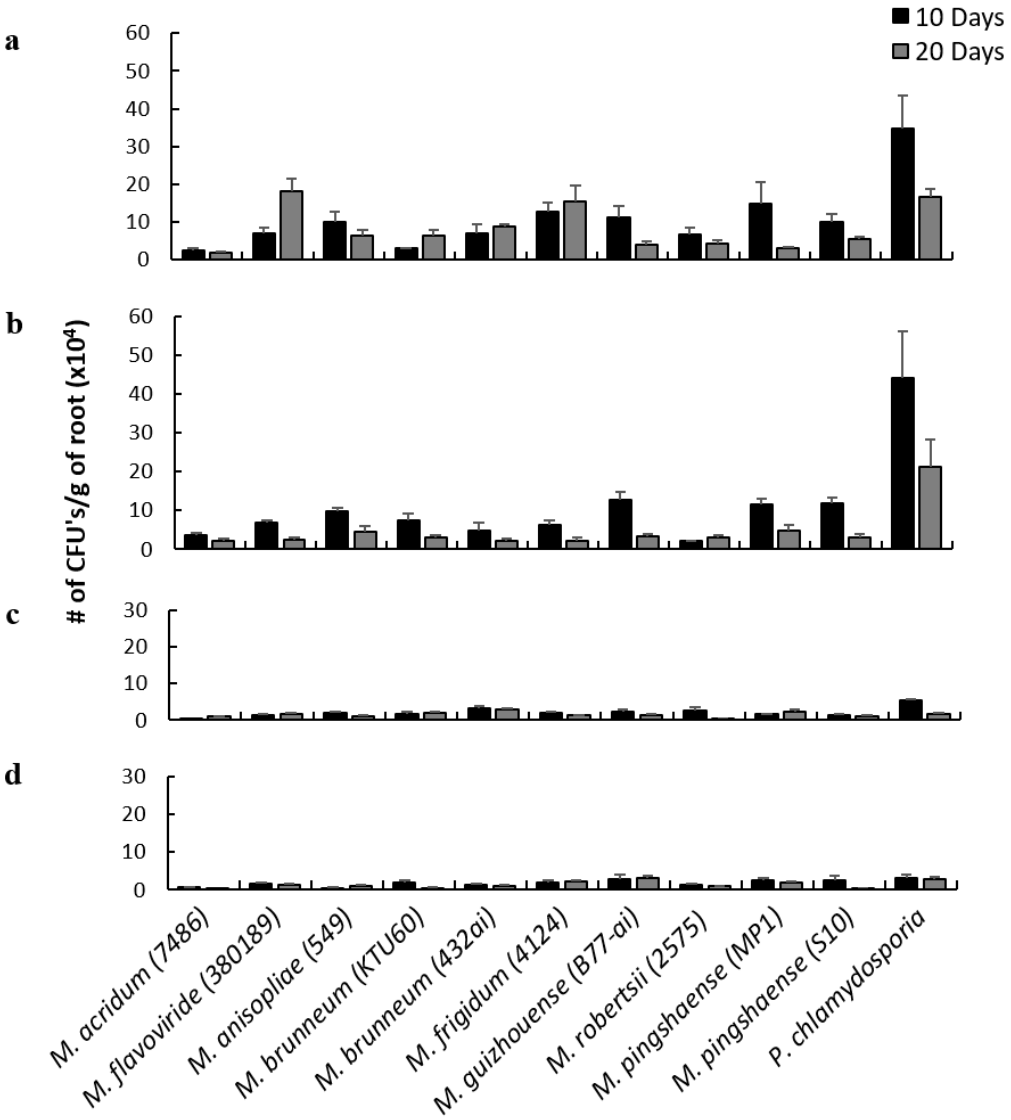


Figure 5.4 Rhizoplane colonization of fungal strains. a. Barley, **b.** Corn, **c.** Beans and **d.** Peas on 10 and 20 days. The error bars represent the standard error for 5 biological replicates

5.4.2.3 Rhizosphere colonization: The persistence of fungal strains in rhizospheric soil was assessed (Figure 5.5). All values are in CFU/g of vermiculite.

In barley (Figure 5.5a), *M. frigidum* ($8.03x10^5$) and *M. pingshaense* (MP1) ($8.72x10^5$) showed greatest rhizospheric association amongst the *Metarhizium* isolates. *M. acridum* ($1.9x10^5$) and *M. robertsii* ($1.59x10^5$) showed the lowest rhizospheric association after 10 days. *M. acridum*, *M. robertsii* and *M. brunneum* (KTU60) showed ($p<0.002$) lower rhizospheric association relative to *P. chlamydosporia*. After 20 days post inoculation, most strains showed a decrease in the persistence in soil except for *M. brunneum* (KTU60).

In corn (Figure 5.5b), no differences in rhizospheric association were observed amongst *Metarhizium* strains after 10 days. *P. chlamydosporia* ($89.67x10^4$) exhibited higher persistence at 10 days relative to the *Metarhizium* ($11.49x10^4$ - $45.93x10^4$) strains ($p<0.01$). However, the recoverable CFU of *P. chlamydosporia* ($43.48x10^4$) from vermiculite was reduced 4-fold after 20 days. Furthermore, no *Metarhizium* strains showed difference in recoverable CFU relative to *Pochonia* except *M. brunneum* (432ai) ($10.78x10^4$) ($p<0.03$).

In beans (Figure 5.5c), *M. acridum* ($3.07x10^4$) showed a decrease ($p<0.01$) in CFU recovery from vermiculite on 10 days compared to *M. pingshaense* (MP1) ($31.09x10^4$), *M. robertsii* ($31.25x10^4$), *M. anisopliae* ($27.64x10^4$), *M. guizhouense* ($30.64x10^4$) and *P. chlamydosporia* ($32.87x10^4$). After 20 days post inoculation, lower colonization was observed for *M. acridum* ($6.03x10^4$) compared with *M. pingshaense* (MP1) ($33.28x10^4$) and *M. guizhouense* ($30.76x10^4$) ($p<0.02$). In addition, *M. robertsii* ($10.68x10^4$) showed lower colonization compared with *M. pingshaense* (MP1) ($33.28x10^4$) ($p<0.02$). *P. chlamydosporia* ($38.84x10^4$) showed higher

colonization relative to *M. acridum* ($6.03x10^4$), *M. frigidum* ($12.89x10^4$), *M. flavoviride* ($13.36x10^4$), *M. pingshaense* (S10) ($16.29x10^4$), *M. robertsii* ($10.68x10^4$) ($p<0.02$) 20 days post inoculation.

On peas (Figure 5.5d), *M. acridum* ($6.08x10^4$) showed lower association compared with *M. brunneum* (432ai) ($25.64x10^4$), *M. flavoviride* ($23.80x10^4$), *M. pingshaense* (S10) ($26.91x10^4$), *M. pingshaense* (MP1) ($24.91x10^4$), *M. guizhouense* ($24.66x10^4$) on 10 days post inoculation ($p<0.01$). In addition, ($p<0.03$) lower colonization was shown by *M. robertsii* ($11.01x10^4$) relative to *M. pingshaense* (S10) ($26.91x10^4$) at 10 days. Among the *Metarhizium* strains, *M. acridum* ($6.08x10^4$), *M. robertsii* ($11.01x10^4$), *M. anisopliae* ($11.66x10^4$) showed differences ($p<0.01$) in CFU recovery from rhizosphere compared to *P. chlamydosporia* ($28.70x10^4$) at 10 days. On 20 days post inoculation, *M. brunneum* (432ai) ($25.79x10^4$) showed higher rhizosphere association relative to *M. acridum* ($3.70x10^4$), *M. robertsii* ($4.26x10^4$), *M. brunneum* (KTU60) ($5.20x10^4$). Among the *Metarhizium* strains, ($p<0.002$) lower colonization relative to *P. chlamydosporia* ($29.46x10^4$) was observed with *M. acridum* ($3.70x10^4$), *M. brunneum* (KTU60) ($5.20x10^4$), *M. pingshaense* (S10) ($4.07x10^4$) and *M. robertsii* ($4.26x10^4$).

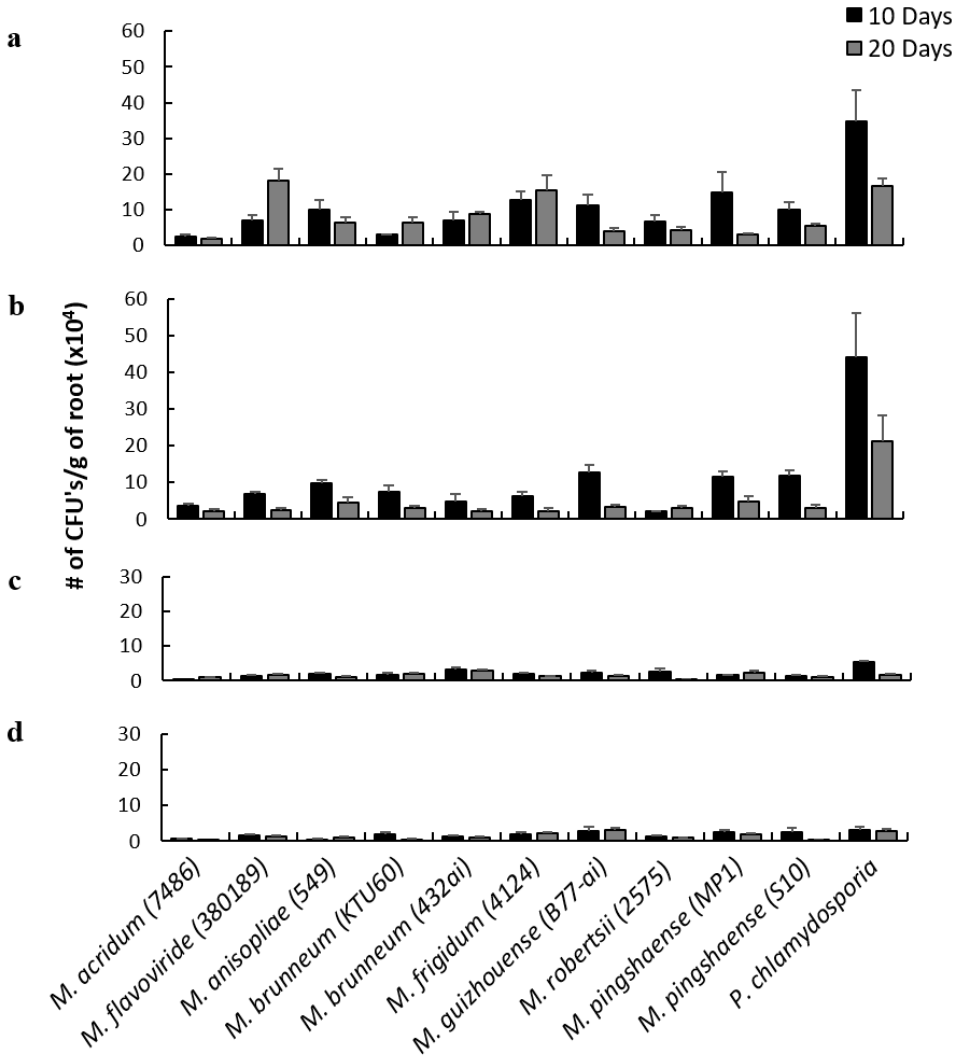


Figure 5.5 Rhizospheric colonization of fungal strains. **a.** Barley, **b.** Corn, **c.** Beans and **d.** Peas on 10 and 20 days. The error bars represent the standard error for 5 biological replicates.

5.4.3 Insect pathogenicity

Insect bioassays confirmed that the specialist strains *M. acridum* and *M. flavoviride* were pathogenic against grasshoppers (Figure 5.6c) while they were not pathogenic against meal worm (Figure 5.6a) or wax moth larvae (Figure 5.6b). Here between 70-80% of *G. mellonella* and *T. molitor* larvae were alive after 15 days. No differences in the pathogenicity were observed against wax moth larvae for *M. anisopliae* 549 (LT50 3.83 ±0.23), *M. brunneum* KTU60 (LT50 3.89±0.11), *M. brunneum* 432ai (LT50 3.68±0.18), *M. frigidum* (LT50 3.98±0.17), M. *guizhouense* (LT50 3.57±0.34), *M. robertsii* (LT50 3.66±0.18), *M. pingshaense* (MP1) (LT50 4.24±0.09), *M. pingshaense* (S10) (LT50 4.00±0.07) (Figure 5.6b & e). Bioassays with meal worm larvae revealed a more diverse pattern of mortality amongst *Metarhizium* strains (Figure 6a & d), with highest virulence observed for *M. anisopliae* 549 (LT50 4.81±0.23), *M. brunneum 432ai* (LT50 4.60±0.21) and *M. guizhouense* (LT50 4.50±0.34). *M. frigidum* was found to be the least virulent with LT50 of 7.66 (±0.17) days. In grasshoppers, all *Metarhizium* strains caused 100% mortality at 4-5 days. Insect bioassays showed that *P. chlamydosporia* showed either no or very low virulence toward the insects tested. Bioassays revealed that between 60-80% of insects were alive after 15 days of topical inoculation.

5.4.4 Conidial hydrophobicity

The surface hydrophobicity of the fungal conidia was evaluated. No differences in conidial hydrophobicity were observed for fungal isolates used in this study. All strains exhibited hydrophobicity indices between 0.88-0.95 (data not shown). No differences in the adhesion to a hydrophobic surface (gel bond film) was observed for *Metarhizium* and *Pochonia* strains (data not shown)

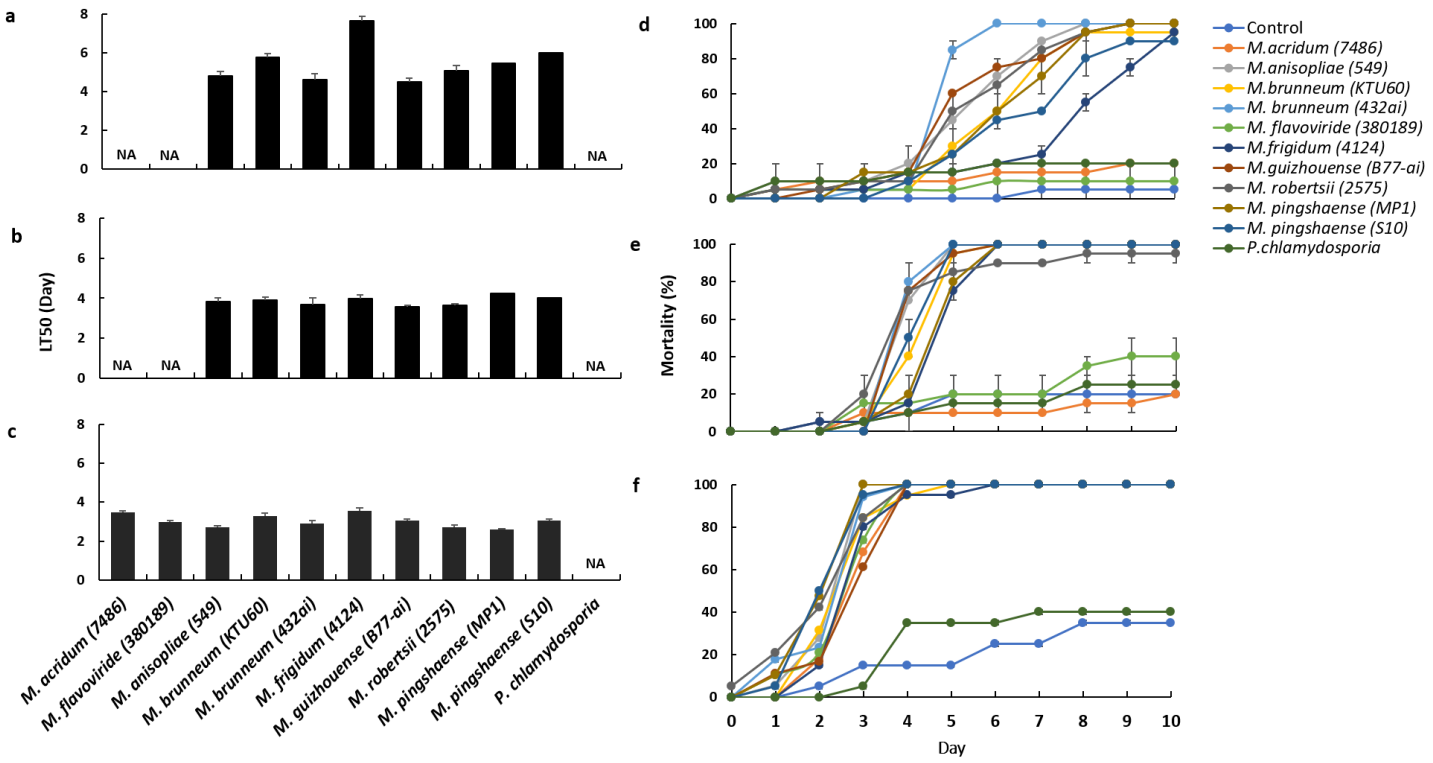


Figure 5.6 Insect bioassay. LT50 values for *Metarhizium strains* and *Pochonia* after topical application to **a.** meal worm, **b.** wax moth larvae, or **c.** grasshopper. Also shown are the mortality curves **d.** meal worm, **e.** wax moth larvae, **f.** grasshopper. LT50 values could not be calculated for strains indicated 'NA' (Not applicable).

5.5 Discussion

In this study, we assessed the rhizoplane and endophytic colonization abilities of ten *Metarhizium* strains and *P. chlamydosporia* with six different plant species. We did not observe any specificity of *Metarhizium* strains towards a particular plant species used in our study with regard to rhizoplane colonization, however, we observed that the *Metarhizium* strains formed endophytic association more readily with monocots compared with dicots. Previous studies have shown that *Metarhizium* species were able to establish endophytic associations with different plant

species as well as the ability to transfer insect derived nitrogen to host plants [6]. We did not observe any endophytic association of *Metarhizium* strains in bean, peas, tomato within 20 days. However, recently we have shown that longer term associations of up to 60 can result in endophytic association with beans [217]. On the other hand, rhizoplane colonization was observed for all *Metarhizium* strains regardless of plant type.

Host plant specificity has been noted in certain ectomycorrhizal fungi, whereas the symbiotic associations of arbuscular mycorrhizal fungi are less host specific [218]. *Metarhizium* were previously shown to form endophytic associations with several plants species including trees [135]. Plant specific rhizosphere associations were reported in *Metarhizium* species. Wyrebek et al. (2011) reported habitat association of three *Metarhizium* species recovered from Ontario, Canada. The study revealed *M. robertsii* to be the most predominant strain recovered from grass roots and wild flowers, while *M. brunneum* and *M. guizhouense* were preferentially associated with shrubs and tree species [94]. The prevalence of specific *Metarhizium* strains associated with certain habitat was reported previously. The phylogenetic diversity of *Metarhizium* population in Brazil revealed *M. anisopliae s.l* as a prominent genotype in savannah habitat as well as agricultural sugarcane fields [210]. *M. robertsii* as the most predominant species in agricultural lands in Maryland, USA, followed by *M. brunneum* as the second most abundant species. However, they also found a two fold increase in *Metarhizium* population in soy bean fields compared to corn fields which was suggested as the influence of rhizosphere or the insect community associated with those plants [211]. The differing abilities of four *Metarhizium* species to colonize perennial plants, shrubs and trees has also been reported [212]. The diversity of *Metarhizium* associated with common crops in Denmark was assessed and *M. brunneum* was the most abundant species associated with cabbage, rye and oats, followed by *M. robertsii* and *M.*

majus and suggests the role of plants in governing fungal populations in soil [209]. The grasshopper specialist, *M. acridum* exhibited low rhizosphere competence [81], while endophytic colonization has been observed under laboratory conditions. There are few studies that show the root colonization ability of the specialist strain, *M. acridum*. In our study, *M. acridum* showed lower endophytic, rhizoplane and rhizospheric interactions relative to other *Metarhizium* strains in both monocots and dicots. In contrast, the other grasshopper specialist *M. flavoviride,* showed comparably greater endophytic colonization in barley and corn, after 20 days. In Europe, *M. flavoviride* is reported as one of the most abundant species recovered from roots or rhizospheric soil in winter wheat and winter oil seed rape [208]. Moreover, *M. acridum* and *M. flavoviride* have shown to transfer insect derived nitrogen to monocot and dicot plant hosts [6]. *M. robertsii* is one the most widely studied *Metarhizium* species in terms of its endophytic ability. Previous studies reported root colonization and the ability to stimulate root hair growth in switch grass and haricot bean, hence its potential as a plant growth promoting fungus [25]. Our study showed that *Metarhizium* can form endophytic associations with barley and corn after 20 days; however, endophytic ability was not observed in dicot plants (bean, peas, tomato and pepper) within 20 days and indicates some level of plant host specificity, however it is unknown whether the association is dictated by the plant or the fungus. A recent study reported low colonization of *M. robertsii* within 28 days on bean roots; however, a more stable association was observed after 60 days. The study also suggested that endophytic colonization of *M. robertsii* could be a transient and a successful or stable colonization could be achieved by long continuous interaction with plants [217].

Previous studies have reported that *Metarhizium* was most often found associated with plant roots or in rhizosphere rather than above ground plant parts [79]. The rhizosphere is a

biologically active soil zone where plants communicate with symbiotic or parasitic microbes. The chemodiversity of the root exudate definitely plays a role in initiating plant-fungal interactions [219]. The spatial localization of root exudates can also affect the interaction with rhizosphere microbes. The pattern of root exudation is not consistent along the root axis. For instance, in the dicot plant *Phaseolus vulgaris*, the influx of nutrients is localized at basal roots compared to adventitious, lateral or tap roots. The exudation of compounds from roots might also have been localized to certain areas which consequently determines the microbial population around that zone [220, 221]. The importance of root hairs as a key for the efficient endophytic colonization of olive roots have been reported in plant growth promoting bacteria such as *Pseudomonas* spp. [222]. Certain compounds in the root exudates could play a role in initiating mutualistic microbial associations. For example flavonoids present in the root of legumes can initiate nodulation genes in *Rhizobium* [223]. *Metarhizium* and *Pochonia* are facultative endophytes and can survive in the soil in close proximity to the roots without endophytically colonizing the plant roots. The difference in the root exudate in monocots and dicots may play a role in the formation of stable symbiotic associations.

P. chlamydosporia has also been used in agricultural fields as a biocontrol agents against root knot and cyst nematodes [224]. Furthermore, it has been described as an endophyte in barley and also exhibited plant-growth promoting properties in barley [225]. In this study, *Pochonia* showed higher colonization in both monocot and dicot plants compared to *Metarhizium* strains. Previous studies suggested the long term endophytic ability of *Pochonia* on plant roots [225]. Furthermore, the presence of *Pochonia* in roots excluded other fungal root colonizers and conferred beneficial effects to their plant hosts [225]. Moreover, *Pochonia* can form stable associations with plant roots through seed inoculation and can successfully maintain colonization

in different soil types including clay and sandy soils [226]. *Metarhizium* and *Pochonia* are phylogenetically related, both are clavicipitacious fungi and often display interkingdom host jumping. Similar to *Metarhizium*, *Pochonia* can parasitize invertebrate hosts, colonize plants endophytically and are able to live saprophytically. While *Metarhizium* species primarily infect insect hosts, *Pochonia* species generally infect nematodes [207].

M. frigidum showed the lowest mortality against meal worm compared with the other generalist strains used in this study. Moreover, among the *Metarhizium* strains the lowest growth rate was observed in *M. frigidum*. However, the CFU root recovery values revealed that slower growth rate has no impact on the root colonization ability of *M. frigidum*. Bischoff et al (2006) identified *M. frigidum* as separate species and a phylogenetically supported lineage within the *M. flavoviride* complex [227]. Initially, *M, frigidum* was described as a variety (*M. anisopliae* var *frigidum*) of *M. anisopliae*, however, Driver et al (2000) reported *M. anisopliae* var *frigidum* as closely related to the *M. flavoviride* clade rather than to *M. anisopliae* [228]. Comparative genomic analysis showed considerable differences in protein families between specialist and generalist species. Moreover, specialization among *Metarhizium* spp. has been proposed as maintained by sexuality and the contraction of protein families, while generalization has resulted from the loss of sexuality and expansion of protein families [87].

The ten *Metarhizium* isolates studied here showed varying degrees of pathogenicity towards insect hosts. *M. acridum* and *M. flavoviride* caused 100% mortality in grasshoppers within 5 days but did not infect Lepidoptera or Coleoptera; while generalist species of *Metarhizium* infected and kill insect hosts belonging to the orders Lepidoptera, Coleoptera and Orthoptera. *Metarhizium* diverged from other Clavicipitacean endophytes (*Epichloe* and *Claviceps*) approximately 100 MYA and acquired the ability to infect insects. Even more recently, ca. 35

MYA *Metarhizium* subsequently diverged in insect host specialization [11]. We observed variation in ability to colonize the rhizoplane and rhizosphere amongst *Metarhizium* species and there was a clear preference for monocots as opposed to dicots. However, regardless of whether the *Metarhizium* species was a generalist or specialist insect pathogen all strains tested showed some ability to associate with plants. Different *Metarhizium* species can form associations with plant roots and the divergence with insect host specificity is not correlated with root colonization or endophytic ability. This suggests that even during the evolutionary divergence in insect host specialization 35 million years ago *Metarhizium* species retained an ecological niche as plant symbionts.

5.6 Conclusions

The present study revealed the abilities of *Metarhizium* species and *Pochonia* to colonize the rhizoplane as well as to endophytically colonize several dicots and monocots. *Metarhizium* species generally colonized monocots better than the dicots. *Pochonia*, was not virulent to any of the insect bioassayed but was a significantly better plant root colonizer when compared to *Metarhizium* species. *M. acridum* and *M. flavoviride* were restricted as grasshopper pathogens but also showed rhizoplane and endophytic colonization. Plant associations were not correlated with fungal growth rates and there were no significant differences in fungal conidial hydrophobicities. During the divergence of *Metarhizium* species as insect specialists all strains tested retained the ancestral ability to colonize plant roots.

5.7 Acknowledgements

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Chapter 6 - General Discussion

In this thesis, we studied three aspects of EIPF (*Beauveria* and *Metarhizium*) and symbiotic association with plant roots. In the first study, we found that the targeted deletion of two hydrophobin (*hyd1* or *hyd2*) gene in *Beauveria* altered the ability of the fungus to colonize plant roots via, altering the expression of MAPK signal transduction pathway. In the second study, we found that the *Metarhizium* genes (*Hyd3*, *Pr1A*, and *Hypo. protein*) which were upregulated during symbiotic association with beans played no role in initiating root colonization. ^{15}N transfer assay and root colonization assay revealed that the nitrogen transporter genes (*MepC* and *Mep2*) contribute to rhizoplane association and mobilizing insect derived nitrogen to plants. In the third and final study, the plant colonization preferences and insect pathogenicity of different *Metarhizium* strains were assayed, and we found that *Metarhizium* species were able to colonize different species of plants irrespective of the generalist or specialist traits.

6.1 Pleiotropic effects of hydrophobin gene deletion in *B. bassiana*

Hydrophobins are small amphipathic proteins that fulfill different functions in the fungal lifecycle including interaction with different biological surfaces and initiating parasitic or symbiotic relationships [214]. The roles of *hyd1* and *hyd2* in the conidial rodlet layer and insect virulence in *B. bassiana* has been previously demonstrated [10]. The results from our study showed that the targeted deletion of *hyd1* or *hyd2* resulted in pleiotropic effects including the reduction of growth rate and conidiation, generation of oxidative stress in growing hyphae, loss of pigment production, and decreased fungal association with bean roots. We also found that the root

colonization ability of double *hyd* (Δ*hyd1*/Δ*hyd2*) mutant was greater than single *hyd* mutants (Δ*hyd1* or Δ*hyd2*). The gene expression analysis showed the involvement of hydrophobins, specifically on stress signaling pathway genes (*Slt2* and *Hog1*). The loss of *hyd1* or *hyd2* also affected the expression of genes involved in virulence (*CDEP*), adhesion (*bad2*) and pigment production (*pks9*). The most striking feature was the greater expression of *Hog1* pathway genes in double mutants compared to single mutants, particularly in bean root exudate. The Hog1 and Slt2 pathways have been identified as stress response pathways, which is critical for several cellular processes including growth, conidiation, virulence and cell wall integrity in *B. bassiana* [64]. Here we suggest that the loss of hydrophobins resulted in a stress to the fungal cell wall which consequently triggers compensatory pathways or stress pathways which was more apparent in the double mutant (Δ*hyd1*/Δ*hyd2*) than single mutants (Δ*hyd1* or Δ*hyd2*). This indicated that the effects of hydrophobins are mediated via the expression of signaling pathways (e.g., MAPK pathway), are manifested in different fungal traits including fungal development and host interaction.

6.2 Ammonium permeases contribute to root association and transfer of insect derived nitrogen to plant hosts

During plant-fungal association, certain fungal partners facilitate the transfer of nutrients such as nitrogen to plants in exchange for carbon [179, 229]. The ability of *M. robertsii* to mobilize nitrogen from dead insects suggests the involvement of nitrogen transporters in root colonization [6]. Two ammonium transporters (MepC and Mep2) were identified in *M. robertsii* and phylogenetic analysis showed close sequence similarities with other plant associating fungi. In this study, the endophytic, rhizoplane, as well as rhizospheric colonization abilities of the Δ*MepC* and Δ*Mep2* mutant strains was tested on barley roots. Compared to the WT, *MepC* and *Mep2* showed

increased rhizoplane colonization on 10 and 20 days respectively. This suggested the involvement of fungal ammonium permeases in root colonization. In addition, the nitrogen transporter mutants showed greater ^{15}N incorporation compared to the WT in MMN treated barley plants after 10 days of growth in the presence of microcosms containing ^{15}N-injected wax moth larvae. Moreover, *MepC* and *Mep2* mutants showed different growth responses in nitrogen source assay, where Δ*MepC* showed severe growth impairment in the absence or low nitrogen conditions. This suggests the differential responses of *Mep2* and *MepC* transporters towards the availability of nitrogen. Ammonia transporters or ammonia permeases are membrane spanning proteins recognized in both plant and fungal species to facilitate the translocation of ammonia or ammonium. The involvement of ammonium permeases in translocating soil derived nitrogen to plant counterparts have been described in plant-arbuscular mycorrhizal (AM) symbiosis [106]. We suggest the enhanced colonization exhibited by *Mep2* or *MepC* would account to the nitrogen catabolite repression. Nitrogen catabolite repression is a regulatory mechanism reported in several fungal species which enable the selective utilization of secondary nitrogen sources in the absence of primary nitrogen sources such as ammonium and glutamine [192]. In certain fungi, this mechanism enhances the hyphal growth. Moreover, this regulatory mechanism maximizes the adaptability of the fungi to the changing environment and studies have shown that ammonium permeases are subjected to nitrogen catabolite repression [193, 194].

RNA-sequencing showed upregulation of *Pr1A, Hypo. protein* and *Hyd3* on *Metarhizium* colonized soy bean roots. However, root interaction assay demonstrated that the loss of any of these genes from *M. robertsii* has no impact in establishing endophytic, rhizoplane or rhizospheric colonization. Our data showed that the deletion of *Hypo. protein* has little effect on insect pathogenicity, whereas the targeted deletion of *Hyd3* and *Pr1A* reduced the virulence of *M.*

robertsii against mealworm. *M. robertsii* exhibits a highly heterogeneous lifestyle, can inhabit soil as saprophytes, as insect pathogens and as endophytes or rhizosphere colonizers. Previous studies demonstrated that different subsets of *Metarhizium* genes were involved in the physiological adaptation to different environmental conditions [71, 81, 184]. The metabolic and biochemical plasticity enable the fungi to survive in different ecological niches [14]. The absence of one gene might be compensated by triggering other associated pathways which nullify the effect of single gene deletion.

6.3 Plant colonization preferences of specialist and generalist *Metarhizium* species

Root colonization data showed that regardless of the insect host range, both specialist and generalist *Metarhizium* spp. can form rhizoplane or rhizospheric association with different plant species while retaining their specialist or generalist insect pathogenic properties. *Metarhizium* is a monophyletic clade that is phylogenetically related to Clavicipitacean endophytes, particularly *Claviceps* and *Epicloe* [87]. *Metarhizium* diverged from other Clavicipitacean endophytes approximately 88-114 MYA and subsequently acquired the ability to infect insects. *Metarhizium* spp. later diverged as generalists or specialist insect pathogens approximately 35 MYA. Comparative genome analysis of specialist and generalist *Metarhizium* spp. have shown that the ability to infect insects is an acquired trait, where generalist was evolved from a specialist which appears to be parallel with insect evolution [87]. Our data suggest even after the adaptation as an insect specialist or generalist, *Metarhizium* species still maintain their ancestral traits as a plant colonizer.

Moreover, *Metarhizium* form a rapid endophytic association with monocot plants than dicot plants in green house conditions. We did not observe any endophytic colonization by *Metarhizium* species even after 20 days in dicot plants. The habitat association or prevalence towards certain plant types have been reported previously in *Metarhizium*, but mostly in field conditions [94, 212]. A recent study has shown that *Metarhizium* forms transient rhizoplane colonization on the initial day and more stable endophytic association were observed after 60 days [217]. The difference in the composition of the root exudate between dicots and monocots may play a role in more spontaneous endophytic colonization by *Metarhizium* on monocots.

6.4 Conclusions and Future directives

This thesis implicated the role of different genes in EIPF during association with plant roots as well as insect pathogenesis. Furthermore, the plant host colonization preferences of specialist and generalist *Metarhizium* strains were also demonstrated. Effective pest management strategies, as well as its efficient use in agriculture, can be established if we understand the specifics of rhizosphere competency of these fungi. *Metarhizium* based formulations are not only an alternative to chemical pesticide but also contribute to improved plant productivity and more importantly improve the soil quality. However, our knowledge on mechanistic as well as the ecological association of this fungal species with plants is still at infancy. A deeper understanding of the underlying processes of the stable association of *M. robertsii* with plant host is necessary. More studies are needed on the genetic aspects of plant-fungal symbiosis, persistence of this fungus in the rhizosphere and also how the population of *Metarhizium* in soil or rhizosphere affects other soil microbiota as well. Furthermore, more studies on fungal inflicted changes to plant immune responses and production of secondary metabolites will broaden the application of this fungus in agriculture.

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